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STUDIES IN FORAGE POISONING

THE RELATION OF B. BOTULINUS TO FORAGE POISONING OR CEREBROSPINAL MENINGITIS IN HORSES

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Forage poisoning, or cerebrospinal meningitis, in horses and mules, has been prevalent for many years in the United States. It was reported by Large (Law, 1911) as early as 1850 in New York State. In 1876, according to Apostolides about 6000 horses, mules and donkeys died of a similar disease in the vicinity of Cairo, Egypt. In 1896, 60 out of 69 horses died in Merseburg from a disease resembling forage poisoning, while the same year in the kingdom of Saxony a total of 1198 horses were attacked, with 76 recoveries. Robertson (Hoare, 1915) in England, described the disease as cerebrospinal fever, and its prevalence has been noted in Norfolk, Essex and London. A similar disease occurring in Austria and Germany among horses is known as Borna disease (Hutyra and Marek, 1916). In 1911 a disease among horses in France, resembling cerebrospinal meningitis, caused heavy losses in the region of Gray.

Federal and state employees have reported numerous sporadic outbreaks of forage poisoning in various localities of the United States, including Iowa, Illinois, Kentucky, Pennsylvania, Maryland, South Carolina and Tennessee, and its prevalence in the middle western and southern states especially has resulted in a heavy loss to the live stock industry. In 1912 it was estimated that 20,000 horses and mules died of cerebrospinal meningitis in Kansas, Nebraska and Missouri. In Kentucky the disease has been more prevalent in some years than in others, but sporadic outbreaks have frequently occurred. The heaviest losses have

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E.M.

accompanied winter feeding, yet the disease has also been observed during the months of July and August.

Numerous microörganisms have been suspected of an etiologic relation to this disease, but the conflicting evidence presented by different investigators has not been substantially or universally accepted; thus the primary cause of forage poisoning in domestic animals has continued a moot question. The frequency of forage poisoning following the ingestion of a variety of feeds visibly contaminated with molds, has led to a rather general belief that organisms of this class are the exciting cause of this disease. Various investigators, however, have failed, experimentally, to incriminate fungi isolated from supposedly poisonous feeds associated with this disease, and certain serious outbreaks of forage poisoning have been observed in horse and mule stock wherein visible cryptogamic contamination was not encountered and the feed to all appearances was sound and wholesome.

The possibility of an etiologic relation of toxic fungi to this disease has not been disregarded in our studies, but certain species resembling *Monascus purpureus* (Went), *Fusarium* sp., *Aspergillus* sp., and *Diplodia zeae*, isolated from poisonous feed and propagated in vitro in nutrient broth and sterile forage extracts, have not been productive of the disease in horses when administered in the feed or injected under the skin, nor of a fatal intoxication in guinea-pigs and rabbits subsequently to ingestion or subcutaneous inoculation. From our preliminary experiments no definite conclusions can be drawn, though the negative results suggest that the relation of cryptogamic contamination of feeds in some outbreaks of this disease is purely secondary in character.

From a bacteriological standpoint, Johne, Ostertag and Grimm (Hutyra and Marek, 1916) independently isolated cocci from the brain and spinal cord of horses fatally afflicted with so-called Borna disease. Ostertag working with a special streptococcus was able to produce death in horses by intraspinal inoculation, but negative results followed other methods of artificial infection. Johne isolated from the fluid of the cerebral ventricles a microorganism which he named *Diplococcus intracellularis-equi*. This

organism resembled the *Diplococcus intracellularis* of the human subject, and when introduced subdurally in horses produced clinical manifestations of cerebrospinal meningitis. Siedamgrotzky and Schlegel isolated a micrococcus from the spinal fluid of a horse, which they considered responsible for the disease, as artificial inoculation of pure cultures proved pathogenic for horses in some experiments. In France the disease is attributed to a bacillus isolated from the brain of affected animals, while in England toxic fungi are considered of primary importance. In this country cocci have been isolated from the nervous systems of animals naturally afflicted; and from an outbreak in Canada, Harrison isolated a coccus from the central nervous system of an affected animal with which he succeeded in reproducing the disease in horses by intraspinal inoculations. Buckley and Shippen (1917) record the successful transmission of the disease in one instance by the intravenous injection of a coccus isolated from the thoracic duct of an affected animal. These findings, however, have not been confirmed independently by other investigators and it is believed that many of the organisms encountered in the encephalon and myel of horses following death from forage poisoning play but a secondary rôle and are possibly to be considered of agonal origin. Furthermore the investigations suggest the possibility of more than one etiologic factor capable of producing similar or indistinguishable clinical manifestations.

In preliminary papers Graham and Brueckner (1917) have reported the isolation of an anaerobic, Gram positive bacillus from an oat hay, and from a corn ensilage. These feeds were originally associated with fatal outbreaks of forage poisoning in horses and mules on Kentucky farms. The two outbreaks were distinctly separate, being located approximately one hundred miles apart, occurring in the winter of 1914–1915, and in the spring of 1917 respectively. It was proved by feeding the oat hay and ensilage that these feeds incorporated the causative factor of the disease independent of other feeds on the premises. The grain threshed from the oat hay, the oat straw, and the water in which the forage of the grain threshed from the straw

had been immersed, all proved capable of producing clinico-anatomic forage poisoning in horses subsequently to ingestion. Chicken excreta obtained from the oat hay disguised in the wholesome feed of a horse produced the disease, while the feeding of chicken excreta from remote sources, similarly disguised in the feed of a horse, resulted negatively. The anaerobic bacilli isolated from the oat hay and ensilage possessed similar biologic properties and later proved to be pathogenically related, in that each engendered in horses the clinical symptoms, recognized in natural outbreaks of cerebrospinal meningitis or forage poisoning, subsequently to feeding 2 mils of broth culture disguised in wholesome feed or as the result of injecting 0.1 mil under the skin. The anatomic alterations observed in fatal experimental cases were further suggestive of the etiologic relation of the bacilli to sporadic forage poisoning.

Following an incubation period lasting from a few hours (twenty-four to thirty-five) to a few days (four to nine) horses receiving the broth culture disguised in wholesome feed manifested symptoms of intoxication, i.e., sudden depression, great muscular weakness, impaired vision, ptyalism, sluggish and imperceptible pulse, paresis of the pharynx and intestinal tract, muscular incoordination, decumbency and marasmus. Animals frequently survived twelve to ninety-six hours following decumbency. The filtered broth culture of the bacilli in question proved fatal, suggesting that the form of cerebrospinal meningitis or forage poisoning under observation was an intoxication per se. Fatal intoxication was further suggested by repeated failure to recover the organism from the blood by cultural methods in the course of the natural and experimental disease, while at death, the brain, spinal cord, liver, heart blood, spleen, kidneys, mesenteric lymphatic glands and suprarenal glands from twenty-six experimental cases engendered by feeding and sub- cutaneous injections were cultured with negative results. In one case the organism was recovered from the stomach contents of a mule which succumbed as the result of ingesting 4 mils broth culture of the bacillus isolated from the oat hay, and in one other instance a similar pathogenic bacillus was isolated from the caecum con-

tents of a horse that had succumbed to drinking water in which the oat hay had been immersed.

Buckley and Shippen (1917), in studying the pathogenesis of *B. botulinus* (a strain isolated from an outbreak of human food poisoning) observed that botulism intoxication, artificially induced in horses, presented clinical and anatomical changes similar to, and even indistinguishable from those recognized in forage poisoning. The morphological and cultural characters of the bacilli isolated from the oat hay and ensilage as observed in broth and in gelatin plate cultures, together with their pathogenic properties for guinea-pigs, horses and mules, suggested a striking relation to *B. botulinus*, which was later corroborated by serological and immunological tests. Serum highly immune to *B. botulinus* proved efficacious in protecting horses against a fatal artificial intoxication induced by the anaerobes isolated from the feeds. Similar immunological tests were conducted upon guinea-pigs with positive results. Serum immune to each strain of bacilli isolated from the feeds proved capable of protecting experimental animals against many times the lethal dose of *B. botulinus*. The agglutinins present in serum highly immune to *B. botulinus* showed a positive agglutinating potency for the bacilli isolated from the forages, while the sera highly immune to each strain of bacilli isolated from the feeds possessed positive agglutinins for the homologous and heterologous strains from the forages, as well as for *B. botulinus*.

In an outbreak of forage poisoning in horses occurring on the McLean farm during the summer of 1916 at Ottawa, Illinois, Prof. H. P. Rusk and Dr. H. S. Grindley of the University of Illinois, proved conclusively by feeding tests that a corn ensilage taken directly from the silo incorporated the etiologic factor, independently of other feeds on this farm. The courtesy extended the writers by Rusk and Grindley afforded an opportunity to survey the premises and observe symptoms and gross anatomic alterations in several experimental horses fatally afflicted from ingesting the silage. The analogy of the clinico-anatomic disease observed in experimental horses on the McLean farm to forage poisoning as observed in Kentucky outbreaks, suggested the

possibility of the ensilage on this farm being contaminated with a toxin-producing microörganism. A bacteriological examination of samples of the ensilage from this silo was therefore undertaken, and it is the purpose of this paper to report preliminary studies. The presence of *B. botulinus* or closely allied organisms was suspected and the anaerobic flora of the ensilage was therefore primarily considered.

Samples of silage. From the surface of the ensilage in the silo, approximately 100 grams of the broken leaves, shattered particles of corn cobs, and shelled corn were collected with sterile forceps and placed in a sterile bottle with tightly fitting ground glass cover. The surface sample (1) was taken from the area being fed daily to experimental horses. A second sample (2) was obtained from the same area after removing six or eight inches of the upper strata. These particles were considered representative of the unexposed ensilage which would be utilized in the course of ingestion experiments projected by Rusk and Grindley. Samples no. 1 (surface ensilage) and no. 2 (subsurface ensilage) were carefully protected from light and retained in the laboratory at a temperature of 45 to 50° F.

Technique of examination. Numerous saprophytic organisms were encountered and different methods of elimination were necessarily employed before adopting a definite procedure. From the samples of ensilage taken from the silo two or three small segments of leaves and other particles representative of the gross sample were placed in a sterile amber glass bottle to which was added 100 mils of sterile distilled water. The contents were gently shaken to wash the feed particles and the bottle placed at a temperature of 45 to 50° F. for twelve to thirty-six hours, following which the coarser particles in the sample were removed by filtering through sterile cotton. 1 mil of the slightly turbid filtrate was then added to 10 mils of glucose pork broth made faintly alkaline to litmus. The heavily inoculated tube was then subjected to a temperature of 80°C. for twenty minutes to eliminate non-spore-bearing organisms. From the heated tube containing a variety of spore-forming organisms representative of the flora on the particles of feed in question, numerous dilu-

tions were planted by platinum loop from tube to tube into faintly alkaline glucose pork broth which had been previously heated to expel the oxygen.

The inoculated tubes were then placed in a partial vacuum, the surface of the medium covered with neutral paraffin oil, or placed in a hydrogen atmosphere and allowed to incubate for five to ten days at a temperature of 22 to 24°C. The nature of the growth could be observed and recorded each day without disturbing the anaerobic conditions. Cultures showing mild gas production, followed by the development of a rancid odor, were stained and examined in cover glass films. A majority of the cultures after staining were not identified, but in some tubes organisms were encountered which morphologically resembled *B. botulinus.*, 0.5 mil of the growth in these tubes was administered to guinea-pigs per os. Cultures proving fatal to guinea-pigs were again heated to eliminate non-sporeforming and vegetative bacteria, and dilutions were planted in neutral glucose gelatin plates. The plates were solidified at 15°C. and glucose gelatin or agar containing glycerine 50% was poured over the surface to insure anaerobic conditions. The plates were then incubated in a partial vacuum at a temperature of 18 to 22°C. Deep colonies developing in the plates were transferred to faintly alkaline pork broth where the morphology and cultural characters were observed.

Numerous non-pathogenic, spore-forming, anaerobic microorganisms were encountered in the silage samples, no. 1 from the surface and no. 2 from the subsurface. A microörganism was isolated from the subsurface sample showing the following characteristics:

Morphology and staining properties. Straight rod-shaped organism, slightly rounded at ends, measuring 0.8 to 1.2 microns by 3 to 6 microns. Slightly motile. Occurring singly and in pairs, rarely longer chains. Intracellular spores located near one end, slightly distending or bulging the wall. Staining evenly with the ordinary anilin dyes. Positive by Gram's method.

Cultural characteristics. Strictly anaerobic, but developing under aerobic conditions in association with *Fusarium* sp. Very

sensitive to light. Optimum temperature for growth from 22° to 25°C. Growing best in media made from meat (pork), but also in decoctions made from forage plants, i.e., corn ensilage, alfalfa, oats and corn, of a faintly alkaline reaction. In media of an acid (+2.5) reaction in association with *Fusarium* sp. favorable growth took place aerobically. The addition of glucose to the medium apparently favored growth, as indicated by energetic gas production and the toxicity of the cultures. Gelatin was liquefied, with an odor of butyric acid in old cultures. Most characteristic development in plates of glucose pork gelatin of a neutral or faintly alkaline reaction. Small, round, yellowish-brown, deep colonies appeared in five to eight days. The colonies under low magnification presented a granular appearance, giving the edge a finely fringed periphery. About the margin of older colonies the area of liquefied gelatin presented numerous fine transparent rays. In the liquefied gelatin the colonies became more or less disintegrated into small irregular granular clumps. Gas was formed, splitting the medium or collecting on the surface of the liquefied areas. The odor of butyric acid was pronounced in old cultures.

The organism produced an extracellular toxin in glucose pork broth and plain pork broth of a faintly alkaline reaction. The filtered broth culture, demonstrated to be sterile by cultural methods, proved fatal to guinea-pigs per os, and following a period of incubation engendered symptoms and death in a mule subsequently to ingestion. Similar symptoms were also recognized in the experimental horses on the McLean farm from ingesting the ensilage and some of the symptoms closely resembled those presented by horses and mules suffering from *B. botulinus* intoxication. It is probable that the soluble toxin is generated even under aerobic conditions in media of an acid reaction in association with *Fusarium* sp. and other saprophytic bacteria since mixed cultures of this character proved fatal to guinea-pigs per os. Filtration experiments were not conducted to demonstrate the presence of toxin in aerobic cultures containing mixed growth.

Pathogenesis. Guinea-pigs proved highly susceptible to broth cultures of the bacillus (ss) isolated from the McLean ensilage. 0.02 to 0.03 mil broth culture per os proved fatal in twenty-four to eighty-four hours to guinea-pigs weighing from 250 to 500 grams each. Depression, roughened coat, slow movement, tense abdominal muscles, ptyalism, loss of appetite, incoördination, muscular weakness, coma, terminating in death, were observed in artificially intoxicated pigs. Two mils filtered broth culture, proved sterile by cultural methods, administered per os resulted in the death of a mule in nine days. The filtrate was ingested, disguised in wholesome feed, and was followed by manifest symptoms of depression on the seventh day. Following a period of incubation, a sleepy, dull, listless attitude developed rapidly, and partly masticated feed with excessive amounts of saliva dropped from the mouth. Paresis of the pharynx became noticeable, accompanied by profuse ptyalism. Muscular weakness and incoördinate movement culminated in decumbency on the eighth day. Complete loss of appetite was noted. Respirations were increased, followng intermittent periods of violent struggling. On the ninth day death occurred in coma. The clinical symptoms observed in the mule strikingly resembled symptoms displayed by animals naturally afflicted from ingesting the silage. The gross anatomic alterations observed upon autopsy were likewise suggestive and resembled changes noted in animals affected with forage poisoning in natural outbreaks of the disease.

IMMUNOLOGICAL AND SEROLOGICAL RELATION OF THE BACILLUS ISOLATED FROM THE ENSILAGE TO B. BOTULINUS

The biological and pathological characters of the bacillus (laboratory index ss) isolated from the McLean ensilage suggested the possibility of securing an efficacious antitoxic serum by injecting sublethal doses into a goat. Serum (A–1) from a goat rendered immune to the bacillus (ss) isolated from the McLean ensilage was administered intraperitoneally to three guinea-pigs in doses of 0.5 mil, 1 mil and 1.5 mils respectively, followed in twenty-four hours by five times the lethal amount of broth cul-

ture of the bacillus (ss) administered by the mouth. The antitoxic serum (A-1) apparently failed to protect the pig receiving 0.5 mil. The other two pigs remained healthy. A control pig receiving a similar amount of the toxin (ss) died in approximately eighteen hours. In a duplicate test similar results were obtained, suggesting that the antitoxic serum (A–1) did not afford complete protection in 0.5 mil doses against five times the lethal dose of (ss) toxin, while 1 mil and 1.5 mils of the serum apparently did afford protection (see chart 1, and fig. 1).

CHART 1

Potency test of goat serum (A-1) immune to bacillus (ss) from ensilage

GUINEA-PIG		INJECTION OF GOAT SERUM (A-1) IMMUNE TO BACILLUS FROM ENSILAGE	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (88) FROM ENSILAGE	RESULTS
Number	Weight			
	grams			
1	200	0.5 mil June 18, 1917	0.1 mil June 19, 1917	Dead 7 a.m. June 28, 1917
2	250	1.0 mil June 18, 1917	0.1 mil June 19, 1917	Released July 2, 1917
3	250	1.5 mils June 18, 1917	0.1 mil June 19, 1917	Released July 2, 1917
4	300	0	0.1 mil June 19, 1917	Dead 7 a.m. June 20, 1917

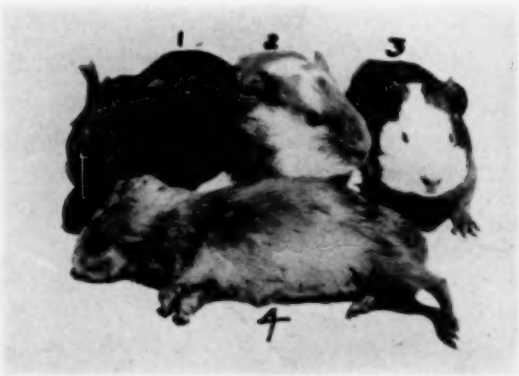

FIG. 1. POTENCY TEST OF GOAT SERUM (A-1) IMMUNE TO BACILLUS (88) ISOLATED FROM MCLEAN ENSILAGE

Control dead in approximately eighteen hours (see table 1).

Following the drawing of serum (A–1) sublethal doses of toxin were administered to the goat, after which an antitoxic serum (B–1 and C–1) was obtained which apparently protected guinea-pigs in doses of 0.5 mil administered intraperitoneally against 0.1 mil of the homologous toxin (see chart 2). Goat serum B–1 and C–1 immune to the bacillus (ss) isolated from the ensilage, administered intraperitoneally in amounts of 0.5 mil, 1 mil and 1.5 mils apparently provided protection to guinea-pigs against a fatal artificial infection of *B. botulinus* administered by the

CHART 2

Potency test of goat serum (B-1 and C-1) immune to bacillus (ss) from ensilage

GUINEA-PIG		INJECTION OF GOAT SERUM (B–1 AND C–1) IMMUNE TO BACILLUS (SS) FROM ENSILAGE	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (SS) FROM ENSILAGE	RESULTS
Number	Weight			
	grams			
1	450	0.5 mil July 10, 1917	0.1 mil July 11, 1917	Healthy. Released July 25, 1917
2	350	1.0 mil July 10, 1917	0.1 mil July 11, 1917	Healthy. Released July 25, 1917
3	400	1.5 mils July 10, 1917	0.1 mil July 11, 1917	Healthy. Released July 25, 1917
4	550	0	0.1 mil July 11, 1917	Dead 7 a.m. July 12, 1917

mouth twenty-four hours later. An unprotected pig succumbed in twenty hours (see chart 3).

Mule serum immune to *B. botulinus*, protected guinea-pigs against five times the lethal amount of broth culture of the bacillus (ss) administered by the mouth. A composite sample of antitoxic serum, consisting of series A, B, C, drawings was injected intraperitoneally into three guinea-pigs in doses of 0.5 mil, 1 mil. and 1.5 mils, respectively. At the end of twenty-four hours each treated pig was given per os, 0.1 mil broth culture of the bacillus (ss). At the same time an untreated pig received a similar amount of broth culture of the bacillus (ss). The pigs receiving the serum remained apparently healthy while the con-

CHART 3

Serological relation of serum immune to bacillus (ss) from ensilage to B. botulinus

GUINEA-PIG		INJECTION OF GOAT SERUM (B-1 AND C-1) IMMUNE TO BACILLUS (SS) FROM ENSILAGE	ADMINISTRATION OF B. BOTULINUS * IN BROTH CULTURE	RESULTS
Number	Weight			
	grams			
1	400	0.5 mil July 2, 1917	0.1 mil July 3, 1917	Healthy. Released July 17, 1917
2	500	1.0 mil July 2, 1917	0.1 mil July 3, 1917	Healthy. Released July 17, 1917
3	550	1.5 mils July 2, 1917	0.1 mil July 3, 1917	Healthy. Released July 17, 1917
4	500	0	0.1 mil July 3, 1917	Dead 7 a.m. July 4, 1917

* B. botulinus N.B.S. strain received from Dr. J. S. Buckley of the Pathological Laboratory, Bureau of Animal Industry, Washington, D. C.

trol pig succumbed in approximately twenty-one hours (see chart 4).

Serum immune to *B. botulinus*, administered intraperitoneally, apparently protected guinea-pigs against many times a lethal amount of the sterile filtered broth culture, or of the unfiltered broth culture of the bacillus (ss) isolated from the ensilage.

CHART 4

Serological relation of serum immune to B. botulinus to bacillus (ss) from ensilage

GUINEA-PIG		INJECTION OF BOTULINUS ANTITOXIN MULE SERUM—A, B AND C	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (SS) FROM ENSILAGE	RESULTS
Number	Weight			
	grams			
1	300	0.5 mil May 19, 1917	0.1 mil May 20, 1917	Healthy. Released May 30, 1917
2	350	1.0 mil May 19, 1917	0.1 mil May 20, 1917	Healthy. Released May 30, 1917
3	350	1.5 mils May 19, 1917	0.1 mil May 20, 1917	Healthy. Released May 30, 1917
4	350	0	0.1 mil May 20, 1917	Dead 7 a.m. May 21, 1917

An animal was subjected to several sublethal doses of *B. botulinus* toxin and at intervals of time serum was drawn from the jugular vein. A composite sample of antitoxic mule serum, series E and F, was injected intraperitoneally into three guinea-pigs in doses of 0.5 mil, 1 mil and 1.5 mils, respectively. Twenty-two hours later these animals received by the mouth 0.1 mil broth filtrate of the bacillus (ss) isolated from the ensilage. A control pig receiving an equal amount of the filtrate per os succumbed in twenty hours, while the serum treated pigs remained apparently healthy and were released after a period of twenty days (see chart 5).

CHART 5

Serological relation of serum immune to B. botulinus to sterile filtered broth culture of bacillus (ss) from ensilage

GUINEA-PIG		INJECTION OF BOTULINUS ANTITOXIN MULE SERUM E AND F	ADMINISTRATION OF FILTERED BROTH CULTURE OF BACILLUS (ss) FROM ENSILAGE	RESULTS
Number	Weight			
	grams			
1	350	0.5 mil May 30, 1917	0.1 mil May 31, 1917	Healthy. Released June 20, 1917
2	350	1.0 mil May 30, 1917	0.1 mil May 31, 1917	Healthy. Released June 20, 1917
3	300	1.5 mils May 30, 1917	0.1 mil May 31, 1917	Healthy. Released June 20, 1917
4	340	0	0.1 mil May 31, 1917	Dead 1 p.m. June 1, 1917

It has been noted that goat serum immune to the bacillus (ss) protected guinea-pigs against 0.1 mil broth culture of the homologous strain per os, and also against 0.1 mil broth culture of *B. botulinus* per os. Goat serum (J) highly immune to *B. botulinus*, injected intraperitoneally into three guinea-pigs in doses of 1 mil, 1.5 mils and 2 mils respectively, apparently provided protection against ten times the lethal amount of the broth culture per os of the bacillus (ss). A control pig receiving a similar amount of the broth culture succumbed in approximately twenty-two hours (see chart 6 and fig. 2).

CHART 6

Serological relation of serum immune to B. botulinus to bacillus (88) from ensilage

GUINEA-PIG		INJECTION OF BOTULINUS ANTITOXIN GOAT SERUM J	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (88) FROM ENSILAGE	RESULTS
Number	Weight			
	grams			
1	600	0.5 mil May 12, 1917	0.2 mil May 13, 1917	Healthy. Released May 28, 1917
2	550	1.0 mil May 12, 1917	0.2 mil May 13, 1917	Healthy. Released May 28, 1917
3	950	2.0 mils May 12, 1917	0.2 mil May 13, 1917	Healthy. Released May 28, 1917
4	500	0	0.2 mil May 13, 1917	Dead 7 a.m. May 14, 1917

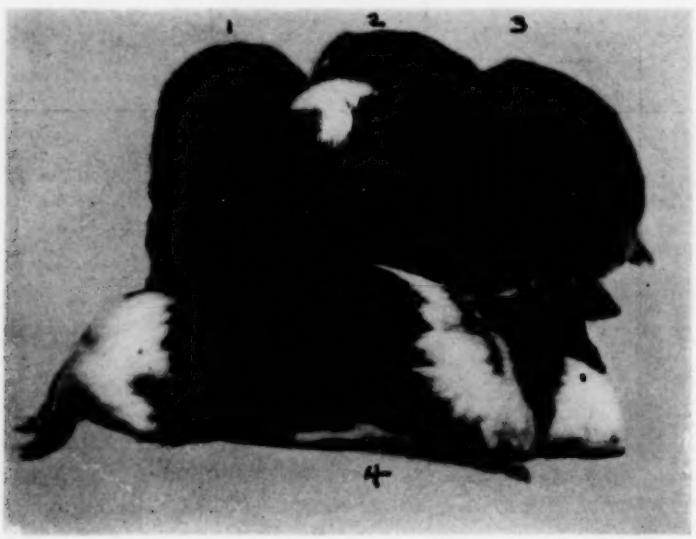

FIG. 2. SEROLOGICAL RELATION OF BOTULISM ANTITOXIN (GOAT SERUM J) TO BACILLUS (88) ISOLATED FROM ENSILAGE

The antitoxin apparently protected the three treated pigs (see table 6).

Similar serological tests were also made upon horses. Blemished and unsound horses were employed in this work, but all animals were serviceable and in good health. Horses 1017 and 1018 received at 4 p.m. May 15, 1917, intravenous injections (40 mils) of mule serum, series E and F, immune to *B. botulinus*. The following day these horses and a control horse, no. 119, were each allowed to ingest 2 mils broth culture of the bacillus (ss) in 1000 grams of wholesome oats (fig. 3). These animals were then supplied with wholesome feed and water each day. On May 19, horse 119 prehended and masticated feed awkwardly, accom-

FIG. 3. HORSES 1017 AND 1018 RECEIVED PROPHYLACTIC DOSE OF SERUM IMMUNE TO B. BOTULINUS, MAY 15, 1917

The following day these horses and a control, No. 119, each received on wholesome feed 2 mils broth culture of the bacillus (ss) isolated from the ensilage.

panied by slight ptyalism. On May 20, a drawn appearance of the flank, profuse ptyalism, muscular weakness, paresis of the pharynx and a stupid, listless attitude were observed, and at 1 p.m. the animal became decumbent. The photograph for figure 4 was made at 2.30 p.m. May 20. In a decumbent position the animal moved the feet violently and suffered from marked dyspnoea. On May 21, decumbency continued, accompanied by marked dyspnoea. The tongue was pendulous and protruding,

feed was refused, and an audible clicking sound was noted in the pharynx. May 22, decumbency continued, ptyalism, mucous discharge from the nostrils, marasmus and enuresis were observed. May 23, death occurred at 7 a.m. Post-mortem examination revealed gross lesions resembling those found in animals naturally afflicted with forage poisoning. Horses 1017 and 1018 appeared dull and exhibited mild transitory symptoms on May 19 and 20, which soon subsided and the animals remained apparently healthy (see chart 7).

FIG. 4. SAME ANIMALS, SHOWING NO. 119 IN A DECUMBENT POSITION
Photographed May 20, 1917 (see table 7).

Agglutination. The agglutinins present in sera highly immune to *B. botulinus* proved active to the homologous strain and to the bacillus isolated from the ensilage in question. Serum immune to the organism (ss) contained agglutinins active to the homologous strain and to *B. botulinus* (see chart 8). Sera immune to a similar bacillus isolated from an oat hay, to a bacillus isolated from the caecum of a horse following death from drinking water in which the above oat hay was immersed, and to a bacillus

CHART 7

Serological relation of serum immune to B. botulinus to the Bacillus (as) isolated from the ensilage

HORSE		INJECTION OF BOTULINUS ANTITOXIN MULE SERUM E AND F	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (as) FROM ENSILAGE	RESULTS
Number	Weight			
	lbs.			
1017	800	40 mils May 15, 1917	2 mils May 16, 1917	Healthy. Released July 2, 1917
1018	830	40 mils May 15, 1917	2 mils May 16, 1917	Healthy. Released July 2, 1917
119	820	0	2 mils May 16, 1917	Dead 7 a.m. May 23, 1917

CHART 8

Agglutination test of B. botulinus (NBS) immune sera from other strains of B. botulinus, B. coli, B. chauvæi and normal sera of sheep, guinea pig, and rabbit

	DILUTION						
	1-100	1-200	1-500	1-1000	1-2000	1-5000	Control
Horse serum immune to NBS strain	++++	++++	++++	+++	+++	++	−
Mule serum immune to NBS strain	++++	++++	++++	++	++	+	−
Horse serum immune to GO6 strain	++++	++++	++++	++++	+++	+	−
Horse serum immune to C91 strain	++++	++++	++++	++	+	−	−
Goat serum immune to SS strain	++++	++++	++++	+++	+++	++	−
Sheep serum immune to GS strain	++++	++++	++++	+++	+++	++	−
Serum immune to *B. coli* species	++	−	−	−	−	−	−
Serum immune to *B. chauvæi*	+	−	−	−	−	−	−
*Normal sheep serum	++++	+++	++	−	−	−	−
*Normal guinea-pig serum	−	−	−	−	−	−	−
*Normal rabbit serum	−	−	−	−	−	−	−

*"Normal" is used to indicate that the animal had not been artificially treated with immune sera or sublethal doses of toxin.

isolated from an ensilage in a remote outbreak of forage poisoning, also contained agglutinins which were active to *B. botulinus* and to the bacillus (ss) isolated from the ensilage (see chart 9). Sera immune to *B. coli*-like organisms and to *B. chauvæi* showed slight and inconsistent agglutinating potency in dilutions of 1–100 to *B. botulinus* and the organism (ss) isolated from the ensilage in

CHART 9

Agglutination test of Bacillus (ss) isolated from ensilage with immune sera from other strains of B. botulinus, B. coli, B. chauvæi, and normal sera of sheep, guinea pig and rabbit

	DILUTION						
	1-100	1-200	1-500	1-1000	1-2000	1-5000	Control
Horse serum immune to NBS strain	++++	++++	++++	++++	+++	++	–
Mule serum immune to NBS strain	++++	++++	++++	++++	+++	+++	–
Horse serum immne to GO6 strain	++++	++++	++++	+++	++	++	–
Horse serum immune to C91 strain	++++	++++	++++	++++	+++	++	–
Goat serum immune to SS strain	++++	++++	++++	++++	++++	+++	–
Sheep serum immune to GS strain	++++	++++	++++	++++	++++	++	–
Serum immune to *B. coli species*	+++	++	–	–	–	–	–
Serum immune to *B. chauvæi*	++	–	–	–	–	–	–
Normal sheep serum	+++	+	–	–	–	–	–
Normal guinea-pig serum	–	–	–	–	–	–	–
Normal rabbit serum	–	–	–	–	–	–	–

question. The sera of the untreated guinea-pig, rabbit and horse showed no agglutinating potency to *B. botulinus* nor to the bacillus (ss) isolated from the ensilage. In one instance serum from an untreated sheep showed mild agglutinating potency to *B. botulinus* in a dilution of 1–200. The presence of agglutinins in the serum of this animal in low dilutions was repeatedly observed (see chart 8 and 9).

DISCUSSION

The extent of forage poisoning in animals resulting from toxin-producing organisms resembling *B. botulinus* is not known, nor is the method by which ensilage and other animal feeds become contaminated well understood. A limited observation suggests that certain conditions in nature are favorable for the development of this type of organism on masses of feed. In this connection it is noted that the three animal feeds from which *B. botulinus* or closely allied organisms have been isolated were stored en masse from two to six months before the contaminated portions were encountered in the feeding operations. Two contaminated feeds in our observations were obtained from silos, while a third, an oat hay, was found stored in a frame barn. The ability of *B. botulinus* and the bacillus (ss) isolated from the McLean ensilage to develop under aerobic conditions with *Fusarium* sp., and also in acid medium, suggests that such organisms might survive in nature under varying conditions. Jordan (1917) states that practically all cases of botulism in human beings have been caused by food that has received some sort of preliminary treatment, such as smoking, canning or pickling. A majority of animal feeds are harvested, cured and stored in bulk for winter feeding, but it is very doubtful if all outbreaks of forage poisoning are associated with stored feed as the clinical disease has been observed in animals on pasture. Outbreaks of botulism in man have been recorded following the consumption of vegetable and fruit products (Dickson, 1916), and the name *botulism* has thus lost its original significance (botulus = sausage) as related to the disease in the human family. Even less appropriate is the term *botulism* as applied to forage poisoning or cerebrospinal meningitis in animals. The term *forage poisoning* appears to be without objection as applied to this type of clinical disease in domestic animals.

The presence on animal feeds of anaerobic bacilli capable of engendering a fatal intoxication when propagated artificially is suggestive of the possible prophylactic or curative value of antitoxic serum in sporadic outbreaks of forage poisoning. In emer-

gency it might be employed as a possible aid to suppress the economic loss from this disease. The value of forage poisoning antitoxin (botulinus antitoxin) in outbreaks of forage poisoning in animals cannot be relied upon until a larger amount of dependable field data is available, but it seems evident from our observations that certain types of this disease may eventually prove amenable to the serum treatment. The use of polyvalent antitoxic serum in outbreaks of forage poisoning, followed by careful observations, might also contribute information relative to the prevalence of *B. botulinus* or closely allied anaerobic toxin-producing organisms encountered in fatal diseases of animals recognized as forage poisoning or cerebrospinal meningitis.

SUMMARY

1. An anaerobic, spore-bearing bacillus (ss) isolated from a corn ensilage, proved to be responsible for an outbreak of forage poisoning at Ottawa, Illinois, engendered clinico-anatomic forage poisoning in a mule following the ingestion of 2 mils of the sterile filtrate broth culture. 0.02 and 0.03 mil broth culture per os generally proved fatal in guinea-pigs in twenty-two to eighty-four hours.

2. A goat injected with sublethal doses of broth culture of the bacillus (ss) isolated from the ensilage developed an antitoxic serum which injected intraperitoneally apparently protected guinea-pigs against a fatal amount of the homologous toxin per os. The protective nature of the goat serum was also observed in guinea-pigs receiving fatal amounts of *B. botulinus* per os.

3. *B. botulinus* antitoxic serum prepared from a mule and injected intraperitoneally into guinea-pigs apparently provided a protection against 10 times the lethal amount of the culture filtrate of the bacillus (ss) isolated from the ensilage and against five times the lethal dose of the unfiltered broth culture.

4. Two horses ingested in wholesome feed 2 cc. broth culture of the bacillus (ss) isolated from the ensilage and were apparently protected by an intravenous injection of botulinus antitoxic serum. A control horse receiving a similar amount of the unfil-

tered broth culture manifested clinical symptoms of forage poisoning and died. Post-mortem examination revealed gross anatomic lesions analagous to some of the gross lesions observed in animals naturally afflicted with forage poisoning.

5. Serum immune to *B. botulinus* possessed a positivea gglutinating potency toward the homologous strain, as well as toward the bacillus (ss) isolated from the ensilage. The agglutinins present in serum immune to the bacillus (ss) isolated from the ensilage were active against *B. botulinus* as well as against the homologous strain.

6. The cultural, morphological, pathological, and serological relation observed between *B. botulinus* and the bacillus isolated from the ensilage in preliminary experiments are suggestive of the possible etiologic significance of *B. botulinus* or closely allied anaerobes in some outbreaks of forage poisoning or cerebrospinal meningitis in horses.

The authors wish to acknowledge the coöperation of Professors Rusk and Grindley of the Illinois Agricultural Experiment Station in affording us an opportunity to study the outbreak of forage poisoning on the McClean Farm and in furnishing us samples of the silage for the investigations here reported.

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AEROBIC SPORE-BEARING BACTERIA IN THE INTESTINAL TRACT OF CHILDREN

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During the last three years a bacteriological investigation of the dejecta of children has been undertaken to determine whether changes in the intestinal flora can be correlated with changes in diet, the results of this investigation being published elsewhere. In the course of this work it became necessary to make a systematic study of the aerobic spore-bearing bacteria which were isolated from the various cases, and the results obtained seem of sufficient importance to merit separate consideration.

Smears made from the dejecta of children when stained by Gram's method, reveal large Gram-positive bacteria which differ markedly in size and shape from the Gram-negative organisms of the intestine. In adults and in many children, they form a relatively unimportant constituent of the faecal flora. In other children, however, especially when a diet of protein milk or buttermilk has been administered, these large Gram-positive organisms are much increased in number and form a considerable proportion of the organisms found on bacterioscopic examination. Together with these forms, spores of various sizes and shapes may be detected; and the large Gram-positive organism are usually, in our opinion, aerobic spore-bearers. Ordinary agar and gelatin plates made from the dejecta reveal only the small opalescent colonies of *Bacillus coli* or *Bacillus alkaligenes* or the large viscid colonies of *Bacillus lactis-aerogenes*, all of which are Gram-negative. If an emulsion of the dejecta be made in broth and heated to 80°C. for twenty minutes and cultures be taken from this at once, or after twenty-four hours in-

cubation, aerobic spore-bearing bacteria, usually Gram-positive, develop in great profusion. The morphology of the various species, particularly that of the members of the mesentericus, cereus and megatherium groups, and the size and shape of the spores are so characteristic and correspond so closely to the morphology of the Gram-positive bacteria and the spores seen in the dejecta as to leave no reasonable doubt of the identity of the two groups. Occasionally when the Gram-positive organisms are in great abundance in the contents of the intestine they appear on plates made directly from the dejecta and here the correspondence between the smears and the cultures is still more striking.

We have now examined the dejecta of over fifty children with the special object of isolating and identifying the aerobic spore-bearers. Several hundred cultures have been studied, the various species being found in the different cases in about the following proportion:

Aerobic spore-bearing bacteria in the dejecta of children

Species	*Number of cases*
Bacillus cereus	32
Bacillus albolactus	17
Bacillus pseudotetanus	9
Bacillus mesentericus	6
Bacillus subtilis	6
Bacillus petasites	2
Bacillus vulgatus	2

From the above table it may be seen that the aerobic spore-bearing bacteria of the intestinal tract in children are the same which have been previously established as the predominant organisms of our environment in Baltimore, in soil, dust, water and milk. It is interesting to note that *Bacillus subtilis* is somewhat rare in the intestines, contrary to the usual statements. This is probably to be explained by the fact that only those organisms corresponding to the *Bacillus subtilis* of Cohn as described by Gottheil (1901), Chester (1901), and Lawrence and Ford (1916), are included in this group, whereas the large Gram-positive elements with oval spores, formerly called *Bacillus subtilis* by many writers, are now referred to the species *Bacillus cereus* and

Bacillus albolactus. The relative frequency of *Bacillus pseudotetanus* should also be noted. This is an aerobic species which is much like the tetanus bacillus in morphology and which produces a round spore in a terminal or sub-terminal position in the vegetative rod. It also resembles *Bacillus putrificus-coli* of Bienstock, one of the putrefactive anaerobes. Since *Bacillus pseudotetanus* is so common, the finding in the dejecta of organisms with round terminal spores, can not in the absence of positive cultures, be regarded as satisfactory evidence that the individuals are harboring the tetanus bacillus; nor can the presence of organisms with this morphology be taken to indicate excessive anaerobic putrefaction. In addition to these well-recognized species, cultures were isolated on five occasions which could not be identified with any previously described in the literature nor with any of the strains kept in the laboratory for purposes of comparison. These organisms have, therefore, been described as new species as follows:

Bacillus badius nov. sp.

This organism is characterized by the beautiful arborescent colonies which it produces on agar plates and in agar stab cultures. These colonies are not unlike those of *Bacillus mycoides* and *Bacillus praus-nitzii* of the mycoides group to which also *Bacillus adhaerens* of Laubach has been referred (1916). It differs from these organisms in its cultural reactions and in its production of a brown pigment, but may be placed in the same group. It has been found in one stool.

Morphology. Vegetative rods long with homogeneous protoplasm and round ends, varying in length from 2.35 to 4.5 microns in twenty-four hour cultures on plain agar (fig. 1). The shorter forms predominate in older cultures. Width about 0.75 micron. On glucose agar the organisms are somewhat swollen (fig. 2). Rarely short chains are formed. Whip forms not seen.

Motility. Active motility in young cultures.

Staining reactions. Gram-positive.

Spore formation. Spores begin to form by the first day on glucose agar but only after four to five days on plain agar. They are oval in shape, subterminal in position. As the sporulating rods degenerate, tags of protoplasm are left, sometimes at one end and sometimes at

both ends of the spore. They measure 1.125 by 1.875 microns. Older spores become free from protoplasmic tags and assume an oval contour with a tendency to squaring of the ends. Such naked spores measure about 0.75 by 1.5 microns (fig. 3).

Agar slant. Rapidly spreading glistening moist growth, buff-colored in young cultures with areas of brown pigmentation. At the end of forty-eight hours the entire growth is brown. The surface remains moist and glistening and the growth is easily scraped from the agar. Later the agar itself becomes pigmented.

Agar stab. Slight growth along line of inoculation definitely arborescent. Surface growth at point of puncture spreading and abundant.

Agar colonies. Buff opaque moist colonies showing great irregularity in size and shape. The edges are fuzzy with beautiful arborizations. Under low power, the colonies are reticulated with fine extensive radial outgrowths.

Litmus glucose agar. Rapidly spreading dry buff-colored growth easily scraped from the medium. No acid. Agar gradually becomes dark blue.

Litmus glucose agar colonies. Colonies similar to those on plain agar with somewhat accentuated arborizations. Dirty white to pale brown in color. No acidity.

Gelatin stab. Slow growth along line of inoculation with rapid cup-shaped surface liquefaction. The liquid gelatin becomes definitely brown.

Gelatin colonies. Irregular brown colonies. No definite arborization. The edges of the colonies are fuzzy and their texture resembles cotton. Rapid liquefaction.

Broth. Rapid production of dense turbidity. No scum. No precipitate. Medium becomes very brown.

Peptone. Fairly rapid growth without scum or precipitate. Medium becomes brown in color.

Potato. Faint dry brown growth.

Litmus milk. Gradual decolorization of the litmus and clearing of the proteins in the milk. Digestion complete in about a week when the fat at the surface becomes brown from the adsorption of the pigment.

Blood serum. Dry buff growth with some tendency to a beaded appearance. It becomes brown in two to three days. Some softening of the serum which gradually dried without definite liquefaction.

Fermentation Tubes. Glucose: Turbidity in bulb and neck. No scum or precipitate. Reaction neutral.

Sucrose: Turbidity in bowl and neck. No scum. No precipitate. Reaction neutral.

Lactose: Turbidity in bowl. No scum. No precipitate. Reaction neutral.

Thermal death point.[1] Spores in broth emulsion survive a pressure of 2½ pounds in the autoclave but are destroyed by 5 pounds pressure. They survive fifteen minutes steaming in the Arnold but are destroyed by thirty minutes exposure.

Bacillus fusus nov. sp.

This organism has been found but once in a specimen of stool planted directly in milk. Morphologically it is similar to the species recently described by Ford (1916), *Bacillus centrosporus*. It differs from it in its reaction to Gram's stain, being Gram-positive, and in certain fundamental cultural features.

Morphology. In twenty-four hours cultures on plain agar the vegetative rods lie singly, are thin and delicate with homogeneous protoplasm (fig. 4). They vary greatly in size and shape because of the early sporulation. They are usually regular in outline with round ends and measure 2.25 to 4.5 microns in length by 0.75 micron in thickness The longer forms predominate. On glucose agar the rods are thicker measuring 1.125 microns and show many long forms, 4 to 6 microns, with whip forms 12 to 20 microns in length (fig. 5). In older glucose agar cultures the whip forms are much longer, often running across the field of the microscope or lying wound up in long coils. They are usually thinner than the short rods, having an average width of about 0.75 microns. In cultures four to five days old involution forms begin to appear, as small heavily staining globular masses measuring 1.5 to 1.875 microns in width by 2.25 to 3.75 microns in length. Shadow forms are common on plain agar.

Motility. Active progressive motility in young broth cultures.

Staining reactions. Gram-positive.

Spore formation. Sporulation begins on plain agar in twenty-four hours but seldom occurs in glucose agar where the involution forms pre-

[1] In determining the thermal death point in the autoclave the pressure is successively raised to the various points indicated, 2½ pounds, 5 pounds, etc., after which the gas is turned off at once and the autoclave allowed to cool. The organisms are thus exposed for only a brief time, usually less than a minute, to the various pressures.

dominate. The spores form in the center of the rods which become pointed as the spore develops (fig. 4). The rods thus take on a spindle-shape (fig. 6). The sporangia are often irregularly swollen with one or both ends pointed. As the spores mature the organisms are bulged, becoming very thick centrally because of the size of the spore. The free spores are large compared with the rods, oval and measure 1.125 by 2.25 microns. They do not retain protoplasmic tags but are provided with a rather thick outer rim of ectoplasm (exine). Great variation is seen in the size and shape of the spores. Some remain oval, others are longer, measuring 1.125 by 3 microns. Others are swollen, measuring 1.875 by 2.25 microns. At times the sides are flattened and the spores become reniform.

Agar slant. Thin white beaded growth never becoming very abundant. Growth at times adherent to the medium.

Agar stab. Faint growth along line of inoculation with a slight bead-like knob at the point of puncture.

Agar colonies. Small pin-point white colonies, smooth and round, with dark centers and pale peripheries. Under low magnification, homogeneous with complete edges.

Litmus glucose agar. Growth more abundant than on plain agar, beaded, without tendency to spread. Easily scraped from the medium. Gradually increasing and persistent acidity.

Litmus glucose agar colonies. Small round white colonies, larger than on plain agar. Under low power usually homogeneous with complete edges but the colonies may show fine reticulation.

Gelatin. Faint growth along line of inoculation. No liquefaction.

Gelatin colonies. Small, irregular or round pale colonies with well defined edges. No liquefaction.

Broth. Slight turbidity and granular precipitate. No scum.

Peptone. Slight turbidity and granular precipitate. No scum.

Potato. Moist, creamy rather abundant growth, at times pale brown in color.

Litmus milk. Gradual reduction of the litmus without digestion of the proteins or coagulation.

Blood serum. Very faint, dry and hardly visible growth.

Fermentation tubes. Glucose: Faint turbidity with a slight granular precipitate in the bowl and neck. No scum. Reaction acid.

Sucrose: Faint turbidity, slight precipitate. No scum. Reaction acid.

Lactose: Faint turbidity with fine granular precipitate. No scum. Reaction acid.

Thermal death point. Spores survive 5 pounds pressure in the autoclave but are destroyed by 7 pounds. They resist steaming in the Arnold for fifteen minutes but succumb to thirty minutes exposure.

Bacillus tritus nov. sp.

This organism has been placed in the simplex cohaerens group chiefly because of the size of the vegetative rods and the method of sporulation. It does not have all of the cultural characteristics of any of the members of this group, and must therefore be regarded as a new species. It has been isolated on but one occasion.

Morphology. The morphology is best observed in forty-eight hour agar culture where the organisms usually lie singly. They are regular in outline, with homogeneous protoplasm and round ends. They measure usually 0.75 by 3.75 microns (fig. 7). Often short almost oval forms occur. On glucose agar the rods are larger averaging 0.75 by 5.25 microns. Whip formation occurs on plain agar and is common on glucose agar (fig. 8). Many of these whips measure 75 microns in length.

Motility. Active progressive motility in young cultures.

Staining reactions. Gram-positive.

Spore formation. The spores are smaller than the vegetative rods. They form early, often appearing in twenty-four hours on plain and glucose agar. They are oval, measuring 1.125 by 1.5 microns and subterminal in position in the vegetative rods. The young spores retain protoplasmic tags of various lengths. As the spores mature these tags are lost, the spores become oval and then measure 0.75 by 1.125 microns (fig. 9).

Agar slant. Thick creamy moist glistening growth made up in young cultures of confluent colonies, about 0.5 mm. in diameter. These gradually coalesce. Older cultures are buff in color and have a tallow-like consistency.

Agar stab. Faint growth along line of inoculation. Translucent buff growth at point of puncture.

Agar colonies. Moist opaque round and irregular surface colonies with dark centers and rather compact but fuzzy peripheries. Deep colonies clear-cut, round or elliptical with occasional fuzzy outlines. Under low power the colonies are reticulated, particularly in the peripheral zones.

Litmus glucose agar slant. Thin dry beaded growth in twenty-four hours with a faint acidity which does not persist.

Litmus glucose agar colonies. Small white almost pin-point surface colonies. Under low power pale, round or irregular with complete edges. Deep colonies punctiform.

Gelatin stab. Faint growth along line of inoculation. No liquefaction.

Gelatin colonies. Rather large irregular brown homogeneous colonies with circumscribed edges. No liquefaction.

Broth. Turbidity and cloudy precipitate which later becomes granular. Fragile pellicle.

Peptone. Faint turbidity and precipitate. No pellicle.

Potato. Faint dry brown growth.

Litmus milk. Faint growth in milk with practically no change in the medium. No acidity. No coagulation.

Blood serum. Shiny wax-like buff growth becoming pale brown. No liquefaction.

Fermentation tubes. Glucose: Faint turbidity in the bulb. Slight sediment. No scum. Reaction neutral.

Sucrose: Appearance the same. Reaction neutral.

Lactose: Appearance the same. Reaction neutral.

Thermal death point. Spores destroyed by 7½ pounds pressure in the autoclave. They survive thirty minutes steaming in the Arnold but are destroyed by exposure for forty-five minutes.

Bacillus lautus nov. sp.

This organism has been placed provisionally in the simplex-cohaerens group, partly because of its morphology and spore-formation and partly because of its cultural characteristics. It differs from the cultures of *Bacillus simplex* and *Bacillus cohaerens* now in the laboratory and from *Bacillus agri*, the species recently described by Laubach and Rice (1916), which is also included in this group. It has been isolated on but one occasion.

Morphology. In twenty-four and forty-eight hour agar cultures the vegetative rods are homogeneous with rounded ends. They vary greatly in size and shape and usually measure 1.5 microns in thickness by 2.25 to 4.5 microns in length (fig. 10). Shadow forms appear early with irregular aggregations of cytoplasm. They are larger than the vegetative rods, measuring 1.875 by 5.25 to 6 microns. Whip forms also appear early (fig. 10) and are very common in old cultures. Involution forms are frequent in old agar growths taking the stain deeply

and measuring 2.25 by 3 microns. On glucose agar the organisms are thicker and often shorter, measuring 1.875 by 2.25 microns (fig. 11), but longer forms predominate. Involution forms are very numerous on old glucose agar.

Motility. Actively motile in young cultures.

Staining reactions. Gram-positive.

Spore formation. Spores are found early on plain agar but later on glucose agar where they seldom appear before ninety-six hours. They are subterminal, wider than the organism from which they spring, and measure about 1.875 by 3 microns. As they mature they lose their protoplasmic rims and become more oval, measuring usually 1.125 by 2.25 microns (fig. 12).

Agar slant. Very thin dry transparent growth without tendency to spread.

Agar stab. Very faint growth along line of puncture with a meager surface growth at point of inoculation.

Agar colonies. Tiny white colonies round and irregular. Under low power granular with complete edges.

Litmus glucose agar. Fairly heavy dry beaded growth. Reaction acid.

Litmus glucose agar colonies. Thin moist irregular and round colonies with dark centers and lighter peripheries. Under low power homogeneous translucent with serrate edges. Acidity marked.

Gelatin stab. Faint growth along line of inoculation. No liquefaction.

Gelatin colonies. Small white colonies with hazy irregular outlines. No liquefaction.

Broth. Early turbidity and late production of a hazy rather flocculent precipitate.

Peptone. Faint growth, slight turbidity, no precipitate.

Potato. Faint moist pale-brown growth.

Litmus milk. Rapid reduction of the litmus, gradual development of acidity which persists. No coagulation. No peptonization.

Blood serum. Diffuse thin pale-brown growth with areas of yellow pigmentation. This develops into a rather dirty brown growth in seven to eight days. No liquefaction.

Fermentation tubes. Glucose: Turbidity in open bulb. No precipitate. No scum. Reaction acid.

Sucrose: Turbidity in bulb. No precipitate. No scum. Reaction acid.

Lactose: Turbidity in bulb. No precipitate. No scum. Reaction acid.

Thermal death point. Spores destroyed in the autoclave at a pressure of 10 pounds. Survive 7½ pounds pressure. Destroyed by live steam in the Arnold sterilizer in thirty minutes.

Bacillus flexus nov. sp.

This organism has been placed in the megatherium group because of the large size of the vegetative rods and the early production of swollen bodies with an aggregation of the cytoplasm in globular masses. In old cultures there is a marked tendency to the development of curved or spiral forms such as are seen in *Bacillus graveolens*. The organism however differs in its cultural reactions from this species and from the other members of the megatherium group described in the literature. It has been encountered but once.

Morphology. On plain agar in young cultures the vegetative rods are large, with squared ends, measuring 1.5 to 1.825 microns in width and 2.25 to 4.5 microns in length with many of the forms equalling 5.25 microns in length (fig. 13). Chain formation is frequent. The protoplasm is homogeneous in the younger elements but rapidly becomes granular as the organisms mature. On glucose agar the larger forms predominate. On both plain agar and glucose agar the rods swell, the protoplasm segments and the elements characteristic of *Bacillus megatherium* and *Bacillus petasites* are seen in great profusion.

These forms measure 1.5 to 2.2 by 4.5 to 6 microns, have little of the usual cytoplasm left but in its place a mass of large highly refractile globular bodies (fig. 14). These are numerous in the various organisms as many as six or eight having been observed in a single bacillus. Shadow forms develop early, often in twenty-four hours. They measure 1.125 to 1.5 by 4 to 10 microns. In older cultures these shadow forms with protoplasmic remnants are very common (fig. 15). In such cultures long chains of organisms twisted on themselves are seen in abundance while the individual elements are often bent or curved and assume bizarre shapes (fig. 15). Whip forms are also common (fig. 16).

Motility. Active progressive motility in young cultures.

Staining reaction. Gram-positive.

Spore formation. Spores are formed early on glucose agar appearing in forty-eight hours but are never plentiful on plain agar. They are

usually terminal or subterminal and resemble megatherium spores. They are wider than the vegetative rod which bulges on sporulation. The free spores are round, measuring 1.5 by 1.5 microns, or oval, measuring 1.125 by 1.5 microns.

Agar slant. Heavy tallow-like growth along the line of inoculation, gradually becoming pale yellowish-brown in color.

Agar stab. Slight development along line of inoculation and tallow-like surface growth.

Agar colonies. Rather profuse dirty-white tallow-like colonies, with circumscribed edges. They vary greatly in shape, some being round and others very irregular. Under low power the colonies appear homogeneous.

Litmus glucose agar. Rather thin growth with pellucid areas, dry, with no tendency to spread. Acidity develops in twenty-four hours and increases.

Litmus glucose agar colonies. Thin transparent moist surface colonies and punctiform deep colonies. Under low power homogeneous with regular edges. Slight acidity produced.

Gelatin. Funnel-shaped liquefaction rather rapid. No scum formation.

Gelatin colonies. Irregular and round dirty-white colonies, homogeneous with circumscribed edges. Rapid liquefaction.

Broth. Turbidity with heavy flocculent precipitate. No scum.

Peptone. Similar to growth in broth but less luxuriant. No scum.

Potato. Raised granular cream-colored abundant growth.

Litmus milk. Slow development of acid and later coagulation. Partial digestion of the casein.

Blood serum. Heavy moist yellow-brown growth. Depression of the growth with slow liquefaction of the serum.

Fermentation tubes. Glucose: Turbidity and faint granular precipitate in bowl and cloudiness in closed arm. No pellicle. Reaction faintly acid.

Sucrose: Turbidity in bowl and neck of tube. No pellicle. Reaction alkaline.

Lactose: Turbidity in bowl and neck. No pellicle. Closed arm cloudy. Reaction acid.

Thermal death point. Spores survive 5 pounds in the autoclave but are destroyed by 10 pounds pressure. They are killed by thirty minutes exposure to steam in the Arnold sterilizer.

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The drawings were made by Mr. W. P. Didusch from smears stained with gentian violet with a Bausch and Lomb microscope No. 12 × eye piece and one-twelfth fluorite oil-immersion objective giving a magnification of 1260 diameters.

FIG. 1. BACILLUS BADIUS. PLAIN AGAR, TWENTY-FOUR HOURS

FIG. 2. BACILLUS BADIUS. GLUCOSE AGAR, TWENTY-FOUR HOURS

FIG. 3. BACILLUS RADIUS. PLAIN AGAR, OLD CULTURE

FIG. 4. BACILLUS FUSUS. PLAIN AGAR, TWENTY-FOUR HOURS

(Batchelor: Aerobic Spore-Bearing Bacteria in Children)

FIG. 5. BACILLUS FUSUS. GLUCOSE AGAR, TWENTY-FOUR HOURS

FIG. 6. BACILLUS FUSUS. PLAIN AGAR, OLD CULTURE

FIG. 7. BACILLUS TRITUS. PLAIN AGAR, FORTY-EIGHT HOURS

FIG. 8. BACILLUS TRITUS. GLUCOSE AGAR, FORTY-EIGHT HOURS

FIG. 9. BACILLUS TRITUS. PLAIN AGAR, FOUR DAYS

FIG. 10. BACILLUS LAUTUS. PLAIN AGAR, FORTY-EIGHT HOURS

FIG. 11. BACILLUS LAUTUS. GLUCOSE AGAR, FOUR DAYS

FIG. 12. BACILLUS LAUTUS. PLAIN AGAR, FOUR DAYS

FIG. 13. BACILLUS FLEXUS. PLAIN AGAR, SIX HOURS

FIG. 14. BACILLUS FLEXUS. GLUCOSE AGAR, TWENTY-FOUR HOURS

FIG. 15. BACILLUS FLEXUS. PLAIN AGAR. OLD CULTURE

FIG. 16. BACILLUS FLEXUS. PLAIN AGAR, OLD CULTURE

NON-LACTOSE FERMENTING BACTERIA FROM POLLUTED WELLS AND SUB-SOIL[1]

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While *B. coli* is generally accepted as a satisfactory index of pollution, it is always desirable to detect the presence of specific pathogens in any suspected material. The methods for isolating such pathogens, though highly developed, are far from satisfactory. In water, one encounters the problem of high dilution, while in contaminated soil, the presence of large numbers of organisms of the *B. proteus* group interferes greatly with the successful detection of typhoid, dysentery, and related types of bacilli. The failure to find the latter organisms does not, therefore, imply their absence, though it may indicate that they are not present in large numbers.

METHODS

The samples of soil and water were collected in sterile wide mouth, glass stoppered bottles. The soil specimens were removed to sterile Petri dishes, thoroughly mixed, and portions of 10 to 25 grams weighed into sterile glass stoppered bottles. Sterile water was then added in the proportion of 2 cc. of water to 1 gram of soil, the bottles shaken vigorously for a few minutes and then allowed to stand until the heavy particles had settled to the bottom. The supernatant suspension was then tested by two or all of the following methods:

[1] Work conducted under a grant of the International Health Board of the Rockefeller Foundation, New York.

Direct plate method. The water and soil suspensions were plated direct on Endo and brilliant green plates, 0.1 cc. being spread on the former, and 0.2 cc. on the latter, respectively. The plates were incubated for twenty-four hours and suspicious colonies fished to Russell double sugar tubes. All the Gram negative non-lactose fermenting bacilli were kept for further study.

Bile enrichment method. Portions of 10, 2, and 0.2 cc. of the soil suspension and 10, 1.0, and 0.1 cc. of the water were inoculated into lactose bile and the tubes incubated for forty-eight to seventy-two hours. A loopful of fluid from all the tubes that showed gas was then spread on both brilliant green and Endo plates, and the suspicious colonies appearing after incubation for twenty-four hours were inoculated into Russell double sugar tubes, and treated as above.

Agglutination with polyvalent anti-coli serum. This method was the converse of that used by Adami and Chopin (1904). Adami and Chopin reported successful results in the isolation of *B. typhi* from water with the aid of antityphoid agglutinating serum. Preliminary tests of this procedure gave unsatisfactory results, because *B. coli* invariably agglutinated together with the *B. typhi* in sufficiently large numbers to crowd out the latter on plates. It seemed that a polyvalent colon agglutinating serum would be more generally applicable, in that it would remove the *B. coli* and leave the specific pathogens, as well as the other non-lactose fermenting bacilli, in the supernatant fluid.

By inoculating 5 different varieties of *B. coli* into a sheep, a serum was obtained that could agglutinate 50 to 60 per cent of a heterogeneous collection of 50 strains of *B. coli*. No agglutinating serum could be obtained against *B. aerogenes* and a type of *B. coli-communior*, which is apparently very close to the capsulated *B. aerogenes*.

This anticolon agglutinating serum was used successfully in the isolation of non-lactose fermenting bacilli from a few samples of soil, water and feces. The procedure was as follows: The serum was added to beef bouillon or peptone broth in a concen-

tration of 1:100. Then 10 cc. of water or the soil suspension or a loop of feces were added to 10 cc. of broth, and the tubes incubated until floculation was visible. (The period of incubation depended, of course, on the number of *B. coli* present at the time of incubation and varied from two to six hours). The tubes were then centrifugalized at low speed for 5 minutes, and the supernatant fluid plated on Endo and brilliant green plates. Two samples of soil, 2 of water, and 2 stools from patients suffering with summer diarrhea yielded successful results with this method. From the stools practically pure cultures of *B. dysenteriae* (Flexner) and the Morgan bacillus respectively were obtained with this procedure.

RESULTS

By the use of the methods outlined above, either singly or in combination, non-lactose fermenting, non-liquefying bacteria were obtained from 15 polluted wells and 14 contaminated subsoil samples. In but one instance were members of this group obtained from both the well and soil of the same premises, but even in this case the organisms possessed different biological and serological characters.

The predominant organism obtained from the wells differed from the one found in the sub-soil. The fifteen wells yielded 2 organisms resembling the acid type of *B. dysenteriae*, 2 strains of the Morgan bacillus no. 1, 9 para-enteritidis, and 2 paracolon bacilli. The soil samples, on the other hand, gave 8 dysentery-like bacillary strains, 7 of which corresponded culturally with the acid and 1 with the Shiga type; 4 para-enteritidis, and 2 paracolon bacillary strains. In only two instances were non-lactose fermenting bacilli obtained from sub-soil at a distance greater than 2 feet from a privy pit. Both were sandy soils examined during the wet period. The distance from the privy in these two cases was 10 feet and the depths were $7\frac{1}{2}$ to $8\frac{1}{2}$ feet respectively.

It is noteworthy that the prevalent type in wells belongs to the paratyphoid or para-enteritidis bacterial group, while that

TABLE 1

The fermentation and agglutination reactions of the non-lactose fermenting bacilli isolated from polluted wells and soil

NUMBER	SOURCE	FERMENTATION REACTION									AGGLUTINATING SERA				TYPE RESEMBLED
		Glucose	Mannite	Sucrose	Xylose	Arabinose	Rhamnose	Salicin	Dulcite	Indol	Enteritidis	Paratyphi B	Hog cholera	Dysentery	
155	Soil	+	+	−	−	+	−	−	−	−				−P	Flexner dysentery
493	Soil	+	+	−	−	+	−	−	−	±				+(100)F	Flexner dysentery
395	Well	+	+	−	−	+	−	−	−	−				+(100)F	Flexner dysentery
112	Soil	+	+	−	−	−	−	−	−	−				−P	
384	Soil	+	+	−	−	−	−	−	−	−				−P	
1552	Soil	+	−	−	−	−	−	−	−	−				−P	Shiga (not toxic)
75	Soil	+	+	+	+	+	−	−	−	−				±(500)F	Strong dysentery
84	Soil	+	+	+	+	+	−	−	−	−				+(100)F	Strong dysentery
512	Soil	+	+	+	+	+	−	−	−	−				+(1000)P	Strong dysentery
437	Well	+	+	+	+	−	−	−	−	−				−P	?
388	Well	+	+	+	+	−	+	−	−	−				±(1000)P	?
60	Soil	+	+	+	+	+	+	−	−	+				±(500)F	?
57b	Well	•	−	−	−	−	−	−	−	+	+1000	−	−	−	Morgan no. 1
318	Well	•	−	−	−	−	−	−	−	+	+1000	−	−	−	Morgan no. 1
286	Well	•	•	−	•	•	•	−	−	−	++100	−	−	−	Enteritidis
30	Well	•	•	−	•	•	•	−	−	−	±100	−	−		Enteritidis
33	Well	•	•	−	•	•	•	−	−	−		−	−		Enteritidis
46	Well	•	•	−	•	•	•	−	−	−	+100	±100	−		Enteritidis
57a	Well	•	•	−	•	•	•	•	−	−	±100	±500	−		Enteritidis
91	Soil	•	•	−	•	•	•	•	−	−	±500	−100	−		Enteritidis
143	Soil	•	•	−	•	•	•	•	−	−	±500	±100	−		Enteritidis
206	Well	•	•	−	•	•	•	−	−	−	±100	+100	−	++(500)P	Enteritidis
213	Soil	•	•	−	•	•	•	−	−	−	−	−	−		Enteritidis
227	Well	•	•	−	•	•	•	−	−	−	+100	−	−		Enteritidis
294	Soil	•	•	−	•	•	•	−	•	−	+100	−	−	±100	Enteritidis
410	Well	•	•	−	•	•	•	•	−	−	±500	++100	−		Enteritidis
417	Well	•	•	−	•	•	•	•	−	−	+1000	−	−		Enteritidis
507	Soil	•	•	−	•	•	•	•	•	+	−	±500	++100	−	Paracoli
461	Soil	•	•	−	•	•	•	•	−	+	−	+100	−		Paracoli
424	Well	•	•	−	•	•	•	•	−	+	−	±500	−	+ colon ser. 500	Paracoli
50	Well	•	•	−	•	•	•	•	•	+			−		Paracoli

Summary of Table I

Total number of specimens..... 29
Number of wells..... 15
Number of soils..... 14

TYPES FOUND IN	DYSENTERY-LIKE BACILLI	PARA-ENTERITIDIS BACILLI	PARACOLON BACILLI	MORGAN BACILLI
Wells.....	2	9	2	2
Soil.....	8	4	2	0

+ = acid without gas.
• = acid with gas.

Note. The salicin positive, indol positive strains are considered as paracolon; the indol negative strains are classed as para-enteritidis. The bacilli fermenting acid without gas are for convenience thrown together into a "dysentery-like" group.

P = polyvalent antidysentery serum.
F = Specific Flexner antidysentery serum.

found in polluted soil resembles the dysentery bacillus. Smith and Moore (1893) found members of the paratyphoid group in the feces of normal cattle, swine, and other domestic animals. Savage (1906–1910) carried out an extensive investigation on the prevalence of non-lactose fermenting bacilli in excreta of man and animals. He reports the presence of para-Gärtner bacilli in large numbers in normal swine, calves, horses, and mice; but claims that in man they are encountered only infrequently and in small numbers. The true Gärtner bacillus was detected by him once only (in human excreta); the others were what he terms the para-Gärtner bacilli, which differed from the type organism in their behavior toward dulcite and salicin and in their agglutination reactions.

The paratyphoid-like organisms isolated from both wells and soil all belong to the para-enteritidis group. Their reactions, source, etc. are shown in the table. They differ from the *B. paratyphi A* in their power to ferment xylose; from the *B. paratyphi B* and *B. enteritidis* in their failure to attack dulcite (with one exception); and from the hog cholera bacillus in their ability to break down arabinose. They also fail to agglutinate in the higher dilutions with *B. paratyphi B*, *B. enteritidis*, or hog cholera sera. Only one strain (294) presents the characteristic

reactions of the *B. enteritidis*, but even this strain is not agglutinated by the serum prepared from a typical strain in a dilution higher than 1:100; although the titre of the serum was well over 2000. All the strains may therefore be considered members of the para-enteritidis group frequently present in excreta of domestic animals, particularly swine (Savage 1906–1910).

The dysentery-like organisms, or, to be more exact, the non-gas producing bacilli, were the forms most frequently isolated from soil and least frequently from wells. None of the strains was true to the dysentery type with respect both to fermentation and agglutination. Two strains were partially agglutinated in a 1:1000 dilution of a polyvalent serum, and two others in 1:500 of either polyvalent or specific Flexner serum. These agglutinated cultures all fermented sucrose and xylose and resembled, therefore, the Strong type. Three strains were agglutinated only in dilution of 1:100 of the polyvalent or specific Flexner serum, although two of them were culturally identical with the Flexner bacillus. The other strains were not agglutinated at all. It is probable that these bacilli bear the same relation to the true dysentery organisms, that the para-Gärtner bacilli do to the B. *enteritidis*. Organisms of this type have been found by Lewis, (1911–1913) Alexander, (1913) and others in stools of normal children and those suffering from diarrhea. As far as I am aware, they have not been reported from other sources; nor have they been shown to be definitely associated with any pathological conditions, although they are reported to be more frequently found in the excreta of children with diarrhea than in normal stools.

Paracolon bacilli are often found in both human and animal excreta. The usual strains ferment salicin and may or may not ferment dulcite. They also produce indol (Savage, 1906–1910) (Kligler, 1914). Their presence is of less significance than that of the other non-lactose fermenting bacilli, since they are as ubiquitous as the typical colon bacilli.

The Morgan bacilli are of interest because they have been definitely associated by the discoverer with the summer diarrheal diseases of children. Organisms of this type were isolated from

two wells but not from the soil. Morgan and Ledingham, (1909) Lewis, (1911–1913) Alexander, (1913) and others report the frequent presence of this bacillus in normal children and its more regular occurrence in children suffering from diarrhea. Morgan and Ledingham also found the bacillus in one cow out of 18 examined but not in the horse; while Lewis isolated it from 5 out of 20 mice. Whether it occurs in other animals is not known.

While it is hard to detect any absolute relations between the non-lactose fermenting bacilli and the sources of soil and water pollution, the fact does seem significant that the organisms most often found in wells are those more frequently encountered in the excreta of domestic animals, while the predominant sub-soil bacteria are more characteristic of human excreta. This relationship between type and host is not definitely established, but is rendered highly probable by the results recorded in the literature. If the indication proves to be a fact, the results reported above suggest a surface origin of the well pollution, and a human origin of the sub-soil contamination, (the adjacent pits were indeed obviously responsible for the latter). A study of the non-lactose fermenting bacilli usually found in the intestines of domestic animals and human beings and their resistance to storage in soil and water might prove of decided value in tracing the source of pollution.

SUMMARY

A study of the biological and serological characters of the non-lactose fermenting bacilli isolated from polluted well waters and contaminated sub-soils is reported. It appears that the type of bacillus predominant in polluted well waters differed from that isolated from polluted sub-soils. The former belonged to the gas producing para-enteritidis group most common in the intestinal tract of swine, cattle, and other domestic animals. The latter, on the other hand, was a non-gas producing form resembling the bacillus found by Morgan, Lewis, and others in the stools of normal individuals, as well as in those of people suffering from diarrhoea.

This relationship between the type of the non-lactose fermenting bacilli and host, though not definitely established, is suggestive. It is possible that a careful study of the non-lactose fermenting bacilli commonly found in the intestinal tracts of man and domestic animals would furnish the basis for an index of the source of pollution.

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ON NITRIFICATION

II. INTENSIVE NITRITE FORMATION IN SOLUTION

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INTRODUCTION

In the first paper from this laboratory dealing with nitrification, (1915) the importance of the problem and the difficulties in its study were discussed. The very great need of pure culture studies on an appropriate synthetic medium was pointed out; but we had not at that time succeeded in consistently producing really vigorously growing cultures of either the nitrite or of the nitrate former. The use of soil extracts in the preparation of the media, which procedure now finds quite extended use in soil bacteriological technique, gave us practically no encouragement, and this finding has been confirmed by subsequent work. Somewhat better growths were obtained on porous media, and among those tried ignited soil seemed to possess the greatest promise. It has some advantages over ordinary soil or mixtures of quartz sand and humus, yet it is not a synthetic substance of known composition and properties, and does not permit us, therefore, to determine with certainty the cause or causes of its beneficial action. Our quest for the conditions or set of conditions which would with certainty give rise to vigorous growths on media of known composition was not abandoned, and after long continued and persistent effort we have been able to stimulate with satisfying certainty luxuriant growths of that one of these forms, the nitrite producer, which was chosen for exhaustive study.

HISTORICAL

The slow and uncertain growth of the organisms of nitrification has been recognized by practically every one of the host of investigators who have studied the process. The reason why a process which takes place so widely, and often so vigorously, in nature is so slow in synthetic laboratory cultures has been the subject of a considerable amount of discussion among modern investigators, but unfortunately little attention has been given to the actual study of the question.

To attempt to review all the literature on methods of cultivating and growing the nitrifying organisms is beyond the scope of this paper. The uncertainty attending the growing of these organisms on synthetic media has led numerous writers to use ordinary soil as a medium, contending that it approaches the "natural" conditions in the field. Because of the one redeeming feature that this medium possessed, that of producing a relatively rapid growth of the organisms, all its undesirable characteristics, such as unknown and variable composition, difficulty of manipulation, and general inadequacy for bacteriological procedures, were overlooked. As an example of extreme views on the comparison of soils and solutions as media for the study of nitrification may be cited again the work of Stevens and Withers (1909) who believed that the physiological processes were more or less fundamentally different in soils from those in solutions, and that the organisms adapted to producing rapid action in solutions, e.g., in sewage effluents, were not identical with those of soil. The fallacy of this type of reasoning has been pointed out and the general superiority of solutions over soil has been emphasized by Löhnis and Green (1914).

In reading the literature on the subject of nitrification one is impressed with the fact that the earlier workers were more aware of the importance of aeration to the process than have been the modern workers. Thus Schlösing and Müntz (1877) found that by passing a current of sterile air through sewage containing calcium carbonate the ammonia contained in the sewage was transformed to nitrate. Thus, we see, that at this

early time the basis was laid for the development of the activated-sludge method of sewage purification. The air bubbling through the sewage was capable of activating a rapid nitrification without adding to the medium any living bacterial cell. These same authors (1879a) pointed out later that aeration was not only useful but essential to nitrification, and that it may be obtained either by bubbling filtered air through a liquid medium or by spreading this medium in very thin layers. Moreover, they emphasized again (1879b) the influence of aeration on the process of nitrification and in a still later paper (1889), Schlösing showed by gas analysis that oxygen is actually taken up during the process of nitrification, and that the quantity of oxygen so consumed bears a constant ratio to the amount of nitrogen nitrified. It is of further interest in this connection to note that Winogradsky (1890a) started his cultures in "flasks with flat bottom of an exceptionally large diameter, in which the liquid formed a shallow layer" and that a little later (1890b), in criticising his own work, he says of these same cultures: "my flasks with a small volume of liquid imperfectly aerated could not be so favorable to the phenomenon of oxidation."

The remarkable work of Boullanger and Massol (1903, 1904) points out again how essential it is in nitrification experiments to obtain good aeration. By the addition of scoria to the solution, and gentle movement at regular intervals so as to renew the film of solution wetting the scoria an increase in the velocity of nitrification was observed. That this hastened nitrification may also be due to some cause other than simple aeration should be considered possible in the light of the recent work bearing on the beneficial action of continued agitation of the liquid cultures of *B. tuberculosis*.

Of special interest and importance in this connection is the work of Lucet (1913) who found that slow and continuous agitation was not only favorable to aerobes but to anaerobes likewise: thus cultures of the organisms of anthrax, cholera, typhoid, diphtheria, glanders, dysentery, tetanus, pseudotuberculosis, leprosy, symptomatic anthrax, erysipelas and cultures of *B. pyocyaneus* were benefited by such treatment.

The explanation of this peculiar phenomenon is sought by Lucet in (1) a better utilization of the medium by a continued contact of new utilizable food with the cells, and (2) a better distribution of the products of cellular activity, which would otherwise accumulate in the immediate vicinity of the cell and would only slowly diffuse.

The intensive nitrification obtained by Müntz and Lainé in recent years, by allowing ammoniacal solutions to flow through peat and carbon black inoculated with nitrifying organisms (1906a, 1906b) should probably be explained both by a greater aeration and an action similar to the one mentioned by Lucet; the same may be said of the work on intermittent and continuous filters by Roland and Gallemand and by Miss Chick.

The effect of the ratio of diameter to depth established by Löhnis (1905) and by Löhnis and Green (1914) is typical of the results to be obtained when the effect of aeration is segregated from the effect of the renewal of environment; unfortunately the importance of the results obtained by these authors is limited by the indefinite significance of such a ratio. Lately Barthel (1910), forgetful of the fact that the practice which he followed had been before recommended by Schlösing and Müntz (see above), published the details of a method for the activation of nitrification by passing a current of air through a relatively high column of solution.

From these quotations, it is evident that the benefit to be derived by the aeration of nitrifying cultures is something which requires no further proof; but while the solution cultures have been discarded by some investigators as unfit, and other media have been suggested to replace them, it is plain that aerated solution cultures should be given a thorough trial.

As mentioned above, and as pointed out by Löhnis and Green, the chief reason why the solution cultures have been discarded by modern investigators, lies in the fact that the workers who discarded them did so after using them under such conditions as would naturally make them unfit for any aerobic culture. In some cases as much as 300 cc. of solutions were used in 1000-cc. Erlenmeyer flasks which arrangement gave a depth of the layer

of solution of several centimeters at complete rest during the whole period of incubation. From the work of Schlösing and Müntz and of Barthel, it would seem that the depth of the solution might be disregarded only if other means are provided for the aeration of the solution.

PHYSIOLOGICAL STUDY OF THE CULTURES

In all our early work care was taken to observe the same precautions which were observed by the early investigators. Very thin layers of solution were used, so as to permit as thorough an aeration as was possible; usually 25-cc. portions of solution were used in 250- or 300-cc. Erlenmeyer flasks, the layers so obtained measuring 3 or 5 mm. in depth.

The standard medium used was that recommended by Omelianski (1899) for the isolation of the nitrite forming organism, and possessed the following composition:

Ammonium sulphate	2.0 grams
Sodium chloride	2.0 grams
Potassium phosphate	1.0 grams
Magnesium sulphate	0.5 grams
Ferrous sulphate	0.4 grams
Water	1000 cc.

sterile magnesium carbonate was added to each culture flask after sterilization. The potassium phosphate used was dibasic.

Nitrite production in this medium under the conditions stated above was slow and unsatisfactory. Indeed in many cases the process failed to take an active course, in some cases failing altogether, in others declining slowly after a period of feeble growth. With cultures of relatively strong action the following behavior was observed.

On October 6, 1916, 25 cc. of the ordinary Omelianski solution in 250-cc. Erlenmeyer flask, which received after sterilization the addition of basic magnesium carbonate, was inoculated with 1 cc. of a pure mother culture.[1]

[1] The term pure culture is used in all our work to denote a culture that when inoculated in bouillon of either *acid* or *alkaline* reaction gives no growth after 10 days and that gives a uniform picture on microscopical observation.

On October 27, after twenty-one days incubation at 30°C. a sister culture was tested and found still to contain some ammonia. On November 1, 1916, after twenty-six days incubation at 30°C., the culture was analyzed and found to contain 8.03 mgm. of nitrogen as nitrite. The original 25 cc. of solution contained 10.6 mgm. nitrogen as ammonia.

Another culture, sister of the one first mentioned, when tested on November 21 was found to contain considerable quantities of ammonia, and when analyzed after twenty-six days incubation at 30°C. was found to contain 7.94 mgm. nitrogen as nitrite.

From what has been stated, it is evident that the action of the cultures was a relatively slow one. It should be noted here that the flasks remained at perfect rest until tested, and then again until analyzed.

Modifications of the original Omelianski solution were unsuccessfully tried. The first attempts were made with the organisms of nitrate formation, using soil extracts in place of water in the preparation of the nutritive solution. No encouraging results being obtained in this direction, attempts were made to modify the solution by the addition of compounds of definite composition. Omelianski solutions for nitrite formation were prepared to which were added small amounts of potassium nitrate, zinc sulphate, potassium iodide, potassium bromide, ammonium-potassium sulphate; as a further precaution, the quantity of ammonium sulphate was reduced to one-half the quantity recommended by Omelianski.

As can be seen in the accompanying table very little if any benefit at all was experienced by such modification. An average of four cultures for each treatment of a nearly pure nitrosomonas gave the results summarized in table 1.

TABLE 1

TREATMENT	MGM. OF NITRITE N IN 25 CC. OF CULTURE, AFTER THIRTEEN DAYS
Ordinary Omelianski solution	3.08
Modified solution	4.51

The use of solutions made so as to contain the iron-aluminium-silico-phosphate, recommended by Kaserer (1911) as adapted to Azotobacter organisms, gave no more encouraging results than the trials reported above.

The addition of dialized silicic acid to the solutions was likewise of no avail.

Failing to obtain the solution of our difficulties by this method of attack attention was directed to the question of oxygen requirements of the "*nitrosomonas.*"

Trials were then made in the use of very large surfaces, and to that end the flat bottomed Fernbach antitoxin flask was adopted. This flask has been used by Barthel in his work on Azotobacter and found satisfactory.

Encouraging results were immediately obtained by the use of these receptacles: certain cultures utilized in four days a dose of 5 cc. of a 5 per cent ammonium sulphate solution, as determined by the disappearance of the ammonia from the nitrifying solutions, the control flasks retaining all or nearly all their ammonia during the same period.

It was then thought that the use of some solid material to be periodically wetted by the solution would enhance the process of oxidation. This is essentially what Boullanger and Massol did,[2] and we therefore searched for the nature and composition of the "scoria" recommended by them. The following paragraph giving a description of the material in question is quoted from a personal communication received from Professor Boullanger: " . . . . . the scoria that we have used are simple residues of the combustion of coal in the grates of generators (black, agglomerated and uncombustible residue) . . . . ."[2]

Since this communication was received after the work in this direction has been initiated, we were unable to repeat exactly Boullanger and Massol's experiments, and ignited soil was used instead of "scoria."

[2] Les scoria que nous avons employées sont de simples residues de la combustion du charbon sous les grilles des generateurs (parties agglomerées, noires, et non combustibles).

Some soil from Paulding County, Ohio, was sifted so as to separate it in its various sizes, taking care not to crush the clumps and agglomerates. These clumps were separated from the finer material and then ignited at a temperature of 700°C.

Three hundred grams of this ignited soil were placed in a Fernbach flask together with 100 cc. of Omelianski's solution and basic magnesium carbonate: after inoculation the flask was inclined on one side so that the solution, after wetting all the soil clumps, collected at the lower part of the flask. This inclination was changed twice daily so that a fresh supply of food came in contact with the bacteria lodging in the soil clumps, at each such period. The result of this trial was quite encouraging. A rapid nitrification ensued, so that for over a week it was necessary to add daily a dose of food corresponding to 1 cc. of 10 per cent sterile $(NH_4)_2SO_4$ solution.

Repetition of this experiment led to the same results. Unfortunately, due to the preliminary nature of these experiments, no quantitative data were collected and it is therefore impossible to state whether all the ammoniacal nitrogen was nitrified or whether a considerable portion of it was volatilized from the alkaline medium. Nevertheless, the results obtained were such as to warrant a study of aeration and a new less complicated line of attack was adopted.

The benefits to be derived from the shaking or agitation of a culture, as indicated by the work of Lucet and of Boullanger and Massol, were used as guiding principles. The enhanced nitrification realized by the last mentioned investigators when they adopted the means of aeration used in the Luxemburg method of vinegar production as well as the results obtained in this laboratory in large Fernbach flasks were kept in mind.

In attempting to obtain strong aeration of our cultures we aimed at an apparatus which would unite the ample contact with oxygen obtained in the German method of vinegar production, with a constant container such as is used in the Luxemburg method of vinegar production. A klinostat on which large Fernbach flasks were placed served the purpose perfectly.

The flat bottoms of the flasks had a surface of approximately 300 sq. cm. and one hundred cc. of solution in these flasks in a horizontal position formed a layer 0.7 cm. deep. The klinostat having an inclination of $\pm 5°$., the flasks were in such a position that the solution would flow to the lower side leaving a layer of ± 1 mm. on the upper half of the flask: i.e., over a surface of ± 150 sq. cm.

The planes of the klinostat performed one revolution every three to five minutes so that this very thin layer was continually shifting to a new surface, and only returned to its original position once every three to five minutes.

By means of this rotary movement, a very slow centrifugal action and a constant mixing of the solution was accomplished so that it was not the same portion of solution which returned on a definite surface at each turn of the klinostat. The accompanying sketch will serve to elucidate the description.

In this work the determination of the transformed nitrogen was accomplished by finding the increase of nitrite nitrogen in the culture after definite periods of time. In every case 5 cc. samples were taken from the cultures, with sterile pipettes, diluted to definite volume in volumetric flasks, and sub-sampled for duplicate analysis. Aliquots of 20 or 25 cc. of this diluted material were analyzed by the starch-iodide method developed in this laboratory (Davisson, 1916) using 0.02 N or 0.01 N. thiosulphate solution.

In order to avoid any personal error in the analytical technique, several samples were analyzed by two persons. Although the absolute results obtained by the two persons varied slightly, the relative differences due to the action of the organisms in the cultures were found to be the same in each case.

The flasks were weighed each time before sampling and the volume of the solution contained on each date determined by subtracting from this weight the tare of the empty flask. The cotton plugs were covered by beakers to reduce to a minimum the possibility of contamination.

The first culture started by this method was inoculated with a nitrite forming organism which had been cultivated in this

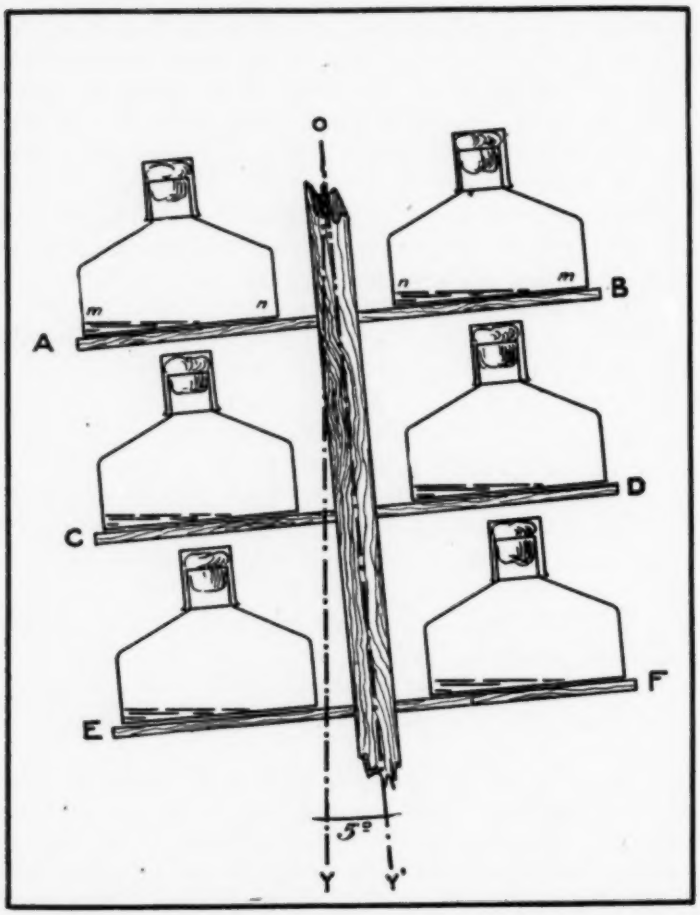

FIG. 1. The klinostat in performing one-half revolution on the axis OY', which is removed from the vertical axis OY by 5°, turns the planes AB, CD, EF, to the opposite position so that the points A, C, E, on the periphery of the planes come to occupy the positions B, D, and F, respectively. The solution then flows from m to n in the flasks on the left side of the figure, and from n to m in the flasks on the right side of the figure. When the klinostat has rotated over an angle of 360°, the solutions assume again the position pictured in the figure.

laboratory for the last three years, and had not been grown in soil for that same period of time.

The results of our first experiment on intensive nitrite formation are shown in table 2. The results are expressed as milligrams of nitrogen oxidized to nitrite per day in 100 cc. of culture.

Leaving aside, for the present, the irregular action of the culture, the rate of oxidation must be considered to be a very rapid one. It surpasses manyfold the action of the ordinary culture which one is accustomed to study and indeed the action of this same organism under the ordinary laboratory conditions. In the light of the above results, many more cultures were started and all gave equally satisfactory results.

TABLE 2

Results on intensive nitrite formation

PERIOD OF INCUBATION	DAILY PRODUCTION OF NITRITE N
First three days of incubation	12.69
Next two days of incubation	10.83
Next one day of incubation	12.43
Next two days of incubation	9.38
Next two days of incubation	14.48
Next three days of incubation	17.70
Next one day of incubation	7.30
Total in fourteen days of incubation	168.92

Table 3 summarizes the action of two other cultures which were started under the same conditions, the results being expressed as before as milligrams of nitrogen oxidized to nitrite per day per 100 cc. of culture solutions.

Here, the weak action during the first four days is due to the small inoculum used and the relatively long period of incubation which caused the amount of nitrogen oxidized during the fourth day to be distributed evenly over a period four times its length.

Leaving aside the irregularities which are due to special treatments, the strong rate of oxidation stands out very prominently. As has already been mentioned, similar results were obtained in every case in which cultures in Fernbach flasks were rotated on a klinostat.

In order to furnish further proof of the possibilities of nitrification when suitable conditions are provided, the results obtained from another series of cultures are summarized in table 4 and graphically shown in figure 2. This set of cultures which was started in order to study the regularity of the phenomenon of nitrite formation under various concentrations of ammoniacal salts, gave very satisfactory results. The results

TABLE 3

PERIOD OF INCUBATION	DAILY PRODUCTION OF NITRITE N	
	Culture I	Culture II
First four days of incubation	0.77	0.94
Next three days of incubation	9.69	7.61
Next two days of incubation	8.31	14.17
Next two days of incubation	9.86	10.15
Next two days of incubation	13.28	18.13
Next four days of incubation	9.86	9.75
Total in seventeen days of incubation	173.93	194.49

TABLE 4

Daily production of nitrite nitrogen (expressed as milligrams of nitrogen per 100 cc. per day)

NUMBER OF CULTURE	DAILY RATE											TOTAL IN 28 DAYS OF INCUBATION
	First 7 days	Next 2 days	Next 3 days	Next 1 day	Next 3 days	Next 1 day	Next 2 days	Next 3 days	Next 1 day	Next 1 day	Next 4 days	
2	2.09	7.90	6.65	12.00	3.96	4.75	12.75	6.47	11.84	9.17	8.09	187.38
3	2.00	9.51	6.45	7.97	4.67	14.74	8.60	7.34	9.75	9.45	11.46	199.05
4	2.45	12.20	4.29	3.06	6.23	11.15	13.77	13.27	11.93	11.09	8.92	221.50

obtained indicate that when a sufficient concentration of ammonium sulphate is present, the nitrite production is very high and that the daily production of nitrite is limited only by the amount of ammoniacal nitrogen present in the culture, and that under uniform conditions of food, the production of nitrite is as uniform as could be expected.

The additions of ammoniacal salts are shown in figure 2.

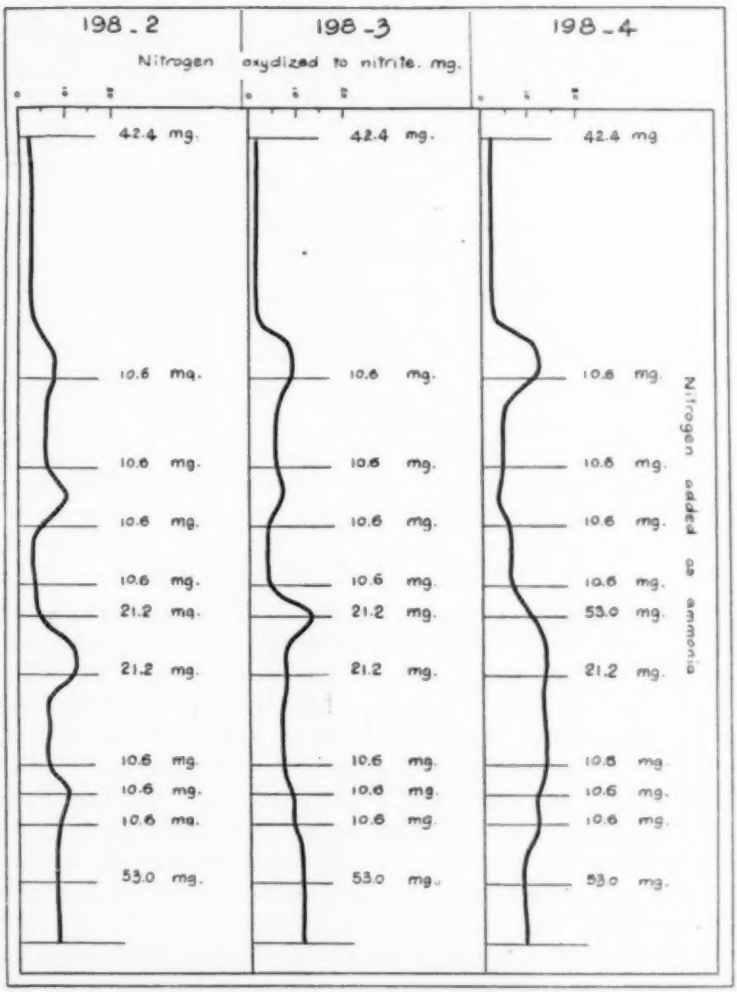

FIG. 2. PRODUCTION OF NITRATE NITROGEN (SEE TABLE 4)

Disregarding now the daily production of nitrite it is possible to obtain an index of the oxidative power of the cultures by expressing it in terms of nitrite production during the whole period of incubation. Table 5, compiled by this method, emphasizes again the strong action of the cultures undergoing a slow movement and strong aeration.

TABLE 5

Oxidative power of cultures undergoing aeration and slow movement

NUMBER OF CULTURE	LENGTH OF EXPERIMENTAL PERIOD	NITROGEN OXIDIZED TO NITRITE
	days	*mgm.*
186-1	14	168.92
194-1	17	173.93
194-2	17	194.49
198-2	28	187.38
198-3	28	199.05
198-4	28	221.50

MICROSCOPIC STUDY OF THE CULTURES

Although the flasks were opened very often and each sampling required some time, during which the cotton plug was removed from the neck of the flask, yet the nitrite formers held the upper hand over any foreign organism which was introduced. Photomicrograph 3 shows the state of purity of one of the cultures when observed by the microscope. Although an infecting form was occasionally observed, yet the intensive transformation of ammoniacal nitrogen to nitrite nitrogen enabled the organism of nitroso-fermentation to grow to such an extent, through the energy thus obtained, that it crowded out the infecting form, rendering its action insignificant.

The beautiful vegetation found in all the flasks studied, makes it quite plain that a strong aeration of the medium with only a moderate movement, not only furthers the oxidative power of the organism of nitrite fermentation, but also stimulates its reproductive power.

The intensive cultures above described were all derived from cultures which in an absolutely pure state grew on silicate jelly forming the colonies pictured in photomicrographs 1 and 2. A full description of the morphology of these organisms will be published in the near future.

DISCUSSION

The results reported above show that the ordinary Omelianski solution for the growth of the nitrite former, will support a very abundant growth of this organism as measured by the production of the by-product of its growth, nitrous acid (or its salts). The conditions under which this luxuriant growth was produced were (1) shallow layers of the solution, (2) slow rotary movement of the culture, and (3) a temperature of 25° to 30°C. The growths reported are far in excess of any yet reported for equal periods of time in solution cultures.

From the results of Löhnis and Green, in which case the ratio of diameter to depth in the solution layers was 90 : 1, and the depth 2 mm., it is seen that even such shallow layers are incapable of supporting a rapid nitrification. Moreover, the results secured by Barthel do not show a much stronger nitrification in aerated than in non-aerated cultures, although the continual bubblings of air through the cultures as used by Barthel may not have been wholly beneficial. It is interesting to note further that Boullanger and Massol reported good results in experiments in which, among other provisions, the cultures were changed in position with respect to a porous substance at the end of every six hours.

It would seem therefore that better aeration may not be the only factor contributing to intensive nitrification, but that change of local environment is in some way associated with it. This assumption is directly in line with the suggestions and postulations of Lucet, and throws a new light on the study of conditions favoring growth in solutions.[3] Whether this change

[3] The present author regrets that he was unable to obtain a recent paper by Otto Meyerhof (Arch. ges. Physiol. **166**, 240–280 (1917). An account of the paper to be found in Physiol Abstr. **2**, 207 (1917) as well as in Chem. Abstr. **11**,

of environment—if indeed it proves to be actually operative—is associated with removal of by-products or better access of mineral nutrients, or possibly some other factor, must be determined by subsequent work.

In conclusion it should be stated that work along these lines is being continued under the general direction of Dr. E. R. Allen, to whom thanks are due for kind advice and criticisms in the preparation of this paper.

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2216, (1917) states that " . . . . under favorable conditions cultures were obtained which in twenty-four hours could oxidize 4 grams $(NH_4)_2SO_4$ per litre to nitrite. . . . ." Unfortunately no mention is made of what actually constitutes the "favorable conditions."

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EXPLANATION OF PLATE 1

PHOTOMICROGRAPH 1. PORTION OF SILICA JELLY PLATE SHOWING COLONIES WITH COLORLESS HALO DUE TO THE SOLUTION OF THE $MgCO_3$

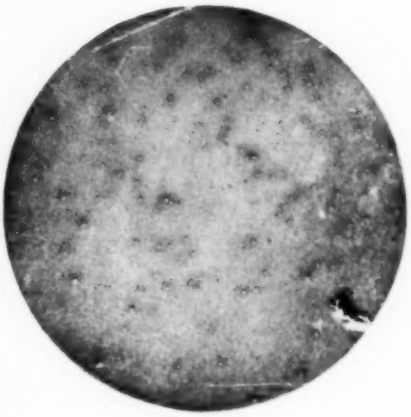
As seen against a black blackground. × 1.5

PHOTOMICROGRAPH 2. A COLONY ON A SILICA JELLY PLATE SHOWING THE LOOSE STRUCTURE

As seen against the source of light. × 77

PHOTOMICROGRAPH 3. PREPARATION FROM A CULTURE IN SOLUTION DURING A PERIOD OF INTENSIVE NITROSO-FERMENTATION

Stained by Winogradsky's malachite-green and gentian-violet method. × 1200



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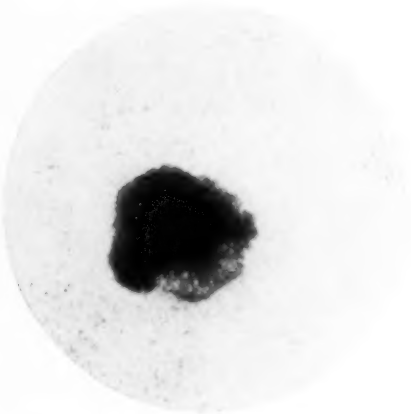

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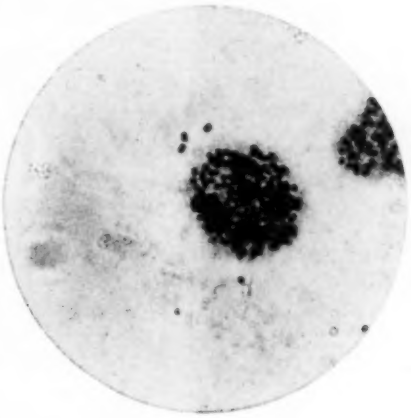

3

STUDIES ON THE BACTERIAL FLORA OF THE MOUTH AND NOSE OF THE NORMAL HORSE

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Received for publication March 5, 1918

The object of this work was to determine the various species of bacteria which are normally present on the mucous membranes of the mouth and nose of the horse. The value of such a study becomes apparent when one considers that horses are subject to many diseases of unknown or doubtful etiology, such as some forms of rhinitis, laryngitis, vesicular stomatitis, etc., and that an endeavor to determine the causative factors in such diseases will invariably involve the elimination of those organisms that are normally present.

Methods. The horses used in this investigation were mares and geldings with perfectly sound and healthy mucous membranes. They included express horses, University Farm horses, and a few horses in the Veterinary Hospital.

Cultures were taken by swabbing, with sterile swabs, the mucous membranes of the hard and soft palates, tongue, cheek, gums, floor of the mouth, and nasal cavity. The swab cultures were used to inoculate tubes of melted agar, which were plated out in the regular manner. Pure cultures were obtained by "fishing" from the colonies thus grown on the plates. Each culture was subjected to the usual cultural and morphological tests, and the determination of species was aided by the use of Chester's "Manual of Determinative Bacteriology," and Matzuschita's "Bakteriologische Diagnostik."

Results. The various bacteria obtained are recorded in the following table:

Organisms present in the mouth and nose of the horse

NUMBER OF ORGANISM	NAME OF ORGANISM	MOUTH—PER CENT OF HORSES IN WHICH IT WAS FOUND	NOSE—PER CENT OF HORSES IN WHICH IT WAS FOUND
1	*B. buccalis*	4	
2	*B. capillaceous*	4	
3	*B. cereus*	4	
4	*B. cloacae*	8	
5	*B. coccineus*	8	
6	*B. coli*	12	
7	*B. cuticularis*		4
8	*B. detrudens*	4	
9	*B. gangraenae*	32	4
10	*B. geminus*	4	
11	*B. liodermos*	4	
12	*B. liquifaciens*	8	
13	*B. magnus*	12	
14	*B. megatherium*	12	12
15	*B. mesentericus*	32	12
16	*B. morbificans*		24
17	*B. prausnitzii*	28	4
18	*B. stellatus*	32	60
19	*B. subtilis*	24	16
20	*B. viscosus*	8	
21	*B. vulgatus*	12	
22	*Mic. aethebius*		4
23	*Mic. aurantiacus*	8	
24	*Mic. citreus*		28
25	*Mic. citreus-granulatus*	8	
26	*Mic. cremoides*	4	
27	*Mic. flavus*	8	
28	*Mic. orbiculatus*	24	20
29	*Mic. ovalis*		8
30	*Mic. pyogenes-albus*	12	12
31	*Mic. simplex*	4	8
32	*Mic. tetragenus*	4	
33	*Molds*	48	64
34	*Ps. pyocyanea*	8	4
35	*Sarcina aurantiaca*	12	12
36	*Sarcina flava*		8
37	*Sarcina lutea*	24	24
38	*Staph. epidermidis albus*	16	16
39	*Streptothrix chromogena*	32	88
40	*Strep. pyogenes*	8	4
41	*Strep. vermiformis*	8	4
42	Not determined (a)	8	

NUMBER OF ORGANISM	NAME OF ORGANISM	MOUTH—PER CENT OF HORSES IN WHICH IT WAS FOUND	NOSE—PER CENT OF HORSES IN WHICH IT WAS FOUND
43	Not determined (b)	8	
44	Not determined (c)	4	
45	Not determined (d)	4	
46	Not determined (e)	4	
47	Not determined (f)	4	
48	Not determined (g)	12	
49	Not determined (h)	8	
50	Not determined (1)		8
51	Not determined (2)		16
52	Not determined (3)		16
53	Not determined (4)		8

It will be observed that twelve organisms are "not determined." i.e., their cultural characteristics did not correspond to those of any species given by Chester or Matzuschita. Most of these bacteria were large, rod-shaped, spore bearers, all but one being Gram positive. They differed but slightly from some of our common saprophytes. To give such forms a new name, and to place them as entirely new species would be entirely unwarranted. They might better be considered, at present, as new strains; the result perhaps, of a slightly different environment.

CONCLUSIONS

1. The nose and mouth of the normal horse harbor a large flora of micro-organisms.

2. The great majority of these organisms are harmless saprophytes.

3. Occasionally present are a few bacteria which may become pathogenic under certain conditions.

BACTERIOLOGICAL NOTES

I. GAS PRODUCTION BY BACT. PULLORUM

II. BACT. PULLORUM INFECTIONS IN ADULT BIRDS

III. RELATION BETWEEN SUCROSE-FERMENTATION AND IMMUNIZING POWER BY B. AVISEPTICUS

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From the Agricultural Experiment Station of the Rhode Island State College, Contribution 243

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I. GAS PRODUCTION BY BACT. PULLORUM

Bact. pullorum (Rettger) is typically a gas-producing organism. Occasionally, however, an anaerogenic strain is observed. Observations made by the writers may throw light on this phenomenon.

In certain tests made in connection with another investigation, certain strains of this organism which had been isolated from eggs or from chicks dying of bacillary white diarrhea or from adult stock, and which had been maintained for some years in the laboratory, were grown in extract and in infusion glucose broths in Smith fermentation tubes. At the same time several strains of *B. gallinarum* (E. Klein), the causative agent of so-called fowl typhoid, were grown in the same manner. The results were as shown in Table 1.

From these data it appears that many strains of *Bact. pullorum* that do not produce gas in extract media may produce gas in infusion media; also that when any amount of gas is produced in extract media a larger amount is produced by the same strain in infusion media. Gas-production in sugar media is apparently strongly influenced by environmental factors independent of the presence of the specific fermentable sugar.

TABLE I

Gas-production by strains of Bact. pullorum and of B. gallinarum in glucose extract broth and in glucose infusion broth

STRAIN NO.	EXTRACT BROTH				INFUSION BROTH			
	24-hour	48-hour	72-hour	96-hour	24-hour	48-hour	72-hour	96-hour
12	0	0	0	0	0	B	B	B
13	B	B	10	10	5	15	20	20
14	0	0	0	0	8	16	20	20
15	B	B	5	5	3	12	15	15
17	0	0	0	0	0	B	3	3
18	0	0	0	0	B	8	10	10
34	0	0	0	0	0	B	3	3
56	0	0	0	0	3	13	15	18
59	0	0	0	0	0	0	0	0
93	0	0	5	5	B	8	12	12
99	0	0	5	5	0	B	8	8
88*	0	0	0	0	0	0	0	0
115*	0	0	0	0	0	0	0	0
116*	0	0	0	0	0	0	0	0
102†	0	0	0	0	0	0	0	0
118†	0	0	0	0	0	0	0	0
162†	0	0	0	0	0	0	0	0

* *B. gallinarum.*

† *Bact. pullorum from infections in adult stock.*

II. BACTERIUM PULLORUM INFECTIONS IN ADULT STOCK

Although cultures of *Bact. pullorum* coming from infected chicks or infected eggs are aerogenic, the writer has been able to demonstrate that anaerogenic strains of this organism do exist. It is a noteworthy circumstance, however, that these strains came in every instance from infections in *adult* stock. The writers have already in another place (1918) presented in detail the circumstances attending an epidemic among adult stock in which *Bact. pullorum* was the causative agent. At the same time reference was made to two other epidemics in adult fowls caused by this organism.

Jones (1913) some years ago discussed an epidemic in adult stock, coincident with a serious infection of chicks on the same farm which was said to be due to *Bact. pullorum*. Since, however at the time of Jones's work, methods were not at hand for the

differentiation of *Bact. pullorum* and *B. gallinarum*, it is not certain which organism was concerned in this case described by Jones. The present writers therefore wish to make it a matter of record that in three widely separated epidemics among adult fowls, each involving a considerable mortality, they have isolated in pure culture, and as the only micro-organism present in cultures from the blood and organs, three bacterial strains conforming to *Bact. pullorum* in all essential respects except that they are not aerogenic. It should be added that the clinical features of the disease, as well as the pathological findings at autopsy, differed in no important respect from those of fowl typhoid. It should also be added that in the ovaries and in the eggs from birds dying in one of the epidemics mentioned, aerogenic strains were also found; but these aerogenic organisms were not found in the other organs of the body, in the heart blood or in the pericardial or pleural exudates. These findings have led the writers, as elsewhere stated, to postulate two types of *Bacterium pullorum:* (1) *Bact. pullorum* α, aerogenic and found only in infections of young stock, or as a latent ovarian infection in adult stock; (2) *Bact. pullorum* β, anaerogenic, and observed only as an agent of active infection in adult stock. Slight fermentative differences between these sub-types may also exist.

An explanation of this diversity of type, together with the difference of selective action in the tissues of the fowl is not at present possible. The questions naturally arise: (1) Are both types present in the intestinal tract of fowls, and does the beta type produce generalized infection because of a difference in aggressivity? Or, (2) may the alpha type give rise to the beta type during the progress of ovarian or intestinal infection, as a result of adaptations, or as a result of a selection of anaerogenic mutants? Is *Bact. pullorum* an instance of *B. gallinarum* in the making?

III. CORRELATION BETWEEN SUCROSE FERMENTATION AND IMMUNIZING POWER OF BACILLUS AVISEPTICUS

One fundamental fermentative characteristic of *B. avisepticus*, and one by which it can be most readily differentiated from members of the fowl typhoid group, is acid-production in sucrose media. The fermentative tests performed by the writers, including a series of fowl cholera strains, showed all, except one, to be sucrose positive. This was strain 52, a culture referred to frequently in earlier publications (1912) as unique in the following points: (1) it is avirulent for adult rabbits, (2) it possesses marked immunizing power against infection with virulent

TABLE 2

Difference of reaction in infusion broth, after two days, of different strains of B. avisepticus. (The percentages give the actual amount of acid produced after the acidity of the control tube has been deducted)

STRAIN NO.	DATE OF TEST		
	January 30, 1918	February 8, 1918	February 18, 1918
48	2.6	2.2	3.5
52	0.2	0.2	0.7
62	2.6	2.5	3.4
83		2.3	3.1
84b		3.3	3.2
91		2.3	2.5
Control	0.5	0.5	0.0

strains.[1] The following tabulation gives the results of titrations of two-day infusion sucrose broth cultures made at different times and in different lots of media (Table 2).

From these data it appears that strain 52 is persistently negative in sucrose broth while the other strains give high acidity. The writers find reported in the literature no other instance of a sucrose-negative strain of *B. avisepticus*. Thus culture 52 is the only sucrose-negative strain described and also

[1] These results of the present writer have been confirmed in several other laboratories, the most complete tests having been made by Gallagher (*Jour. Amer. Vet. Med. Assn.*, 1916–17, **50**, 708–728).

the only immunizing strain. Whether this relation of biochemical features is coincidental or whether it is of some definite significance in immunity-production remains a question.

SUMMARY

1. Gas production by *Bact. pullorum* may depend upon whether the cultures are grown in glucose extract or in glucose infusion broth. Propagating cultures for many years on artificial media does not cause them to lose their gas-producing ability.

2. Although *Bact. pullorum* isolated from epidemics of bacillary white diarrhea in young chicks, or from infected eggs, is aerogenic, there exist also anaerogenic strains which, in all the cases in which they have been observed, have been isolated from adult fowls experiencing acute or sub-acute infections simulating fowl typhoid in both clinical symptoms and pathological alterations in the tissues.

3. Since the anaerogenic quality is thus positively correlated with source, the writer proposes tentatively to postulate for *Bact. pullorum* two sub-species: (1) *Bact. pullorum* α, aerogenic, pathogenic for chicks; and (2) *Bact. pullorum* β, anaerogenic and pathogenic for adult stock only.

4. *B. avisepticus* typically produces an acid-fermentation in sucrose. Among several strains examined, however, one (strain 52) was negative for this sugar. In this instance the absence of sucrose fermentation was correlated with the ability to produce in rabbits resistance against powerful infections with virulent cultures, an ability possessed by no other strain. The question is raised as to the significance of the fermentative reaction in relation to immunity production.

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